DELIVERY OF A KYOTORPHIN ANALOG INTO THE CENTRAL NERVOUS SYSTEM BY MOLECULAR PACKING AND SEQUENTIAL METABOLISM: DESIGN, SYNTHESIS AND PHARMACOLOGY

By

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DEDICATED TO MY MOTHER

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ABBREVIATIONS

Ala (A):	alanine
Arg (R):	arginine
BBB:	blood-brain barrier
BTRA:	the brain-targeted redox analog for Tyr-Lys (a kyotorphin analog)
Boc:	*tert*-Butyloxycarbonyl
CDS:	chemical delivery system
CDS-P:	the chemical delivery system for Tyr-Lys with a single proline as the spacer
CDS-PA:	the chemical delivery system for Tyr-Lys with Pro-Ala as the spacer
CDS-PP:	the chemical delivery system for Tyr-Lys with double prolines as the spacer
CNS:	central nervous system
DADLE:	D-Ala^2-D-Leu^5-enkephalin
DCC:	dicyclohexylcarbodiimide
DCU:	dicyclohexylurea
DIEA:	diisopropylethylamine
DMAP:	4-dimethylaminopyridine
DMF:	dimethylformamide
DMSO:	dimethylsulfoxide
DNP:	dinitrophenyl
Et_3N:	triethylamine
FAB:	fast atom bombardment ionization
g:	gram
Fmoc:	9-florenylmethoxycarbonyl
HOBt:	N-hydroxybenzotriazole
i.v.:	intravenously
KAYK:	Tyr-Lys (a kyotorphin analog)
KAYK-CDS:	the chemical delivery system for Tyr-Lys (a kyotorphin analog)
KTP:	kyotorphin
KTP-CDS:	the chemical delivery system for kyotorphin
HPLC:	high performance liquid chromatography
LH:	luteinizing hormone
Lys (K):	lysine
ml:	milliliter
mmol:	millimole
MS	mass spectrometer

Nys:	the redox analog of the natural diamino acid lysine
Nys^+:	the quarternized form of the redox analog of the natural diamino acid
PAG:	the periaqueductal gray
PG:	propylene glycol
POMC:	proopiomelanocortin
Pmc:	2,2,5,7,8-pentamethylchroman-6-sulfonyl
Pro (P):	proline
s.c.:	subcutaneously
TFA:	trifluoroacetic acid
TLC:	thin layer chromatography
TRH:	thyroid-releasing hormone
Tyr (Y):	tyrosine
VTA:	ventral tegmental area

Abstract of Dissertation Presented to the Graduate School
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Kyotorphin (KTP -- L-Tyrosyl-L-Arginine) is an endogenous neuropeptide which exhibits analgesic action through induction of the release of enkephalin in the brain. Tyr-Lys (KAYK) is a kyotorphin analog which has almost the same analgesic effect as kyotorphin. The peptide sequence, Tyr-Arg or Tyr-Lys, was incorporated into brain-targeted delivery systems to achieve their brain delivery for potential pain-relief treatment. These brain-targeted delivery systems disguise the peptide nature of Tyr-Arg or Tyr-Lys and provide necessary lipophilic functions to penetrate the blood-brain barrier (BBB) by passive transport. Through sequential enzymatic bioactivation, the target

peptide, Tyr-Arg or Tyr-Lys, is retained in the brain and released in pharmacologically significant amounts *in situ*.

A brain-targeted chemical delivery system for Tyr-Arg (KTP-CDS) was synthesized. It was found that the Boc method for the synthesis of KTP-CDS is not practical. The Fmoc method is suitable to synthesize KTP-CDS. However, the KTP-CDS proposed has to be further modified to become a complete molecular package for brain delivery. The modification was done by replacing arginine with lysine. The replacement avoided the difficulties of dealing with the ε-NH_2 group of arginine. A series of brain-targeted chemical delivery systems for Tyr-Lys (KAYK-CDS) and a the brain targeted redox analog for Tyr-Lys (BTRA) was synthesized by a stepwise procedure in solution starting with esterification of lysine derivatives with cholesterol and followed by coupling of the respective peptide cholesteryl ester with Boc- and Fmoc-amino acids using the DCC/HOBt method.

Analgesic actions of the CDS's and BTRA of Tyr-Lys were observed by increased tail-flick time of rats, which indicated the successful delivery of Tyr-Lys into the brain. All CDS's and BTRA showed analgesic activity upon systemic administration, the extent of which depends on the number and the nature of the spacer.

CHAPTER 1
INTRODUCTION

The Neuropeptides and Neuromodulation

Over the past 30 years there have been two developments in neuroscience which have fundamentally influenced research on neural substrates of behavior. The first was anatomical demonstration of the brain's monoaminergic pathways, followed by extensive behavioral and pharmacological investigation of the nature and function of these neurons. These studies fostered notions about the role of neurotransmitters in the regulation of behavior, as well as forming the basis of many of the current biological theories of mental illness. The second important development was the discovery and characterization of peptide-containing neurons in the brain.

Extracellular chemical messenger molecules may be broadly classified into three categories: ions (Na^+, K^+, Ca^{2+}, Cl^-, etc.), amines and amino acids (catecholamine, serotonin, histamine, glutamic acid), and peptides. Peptides are defined as anywhere in size from two amino acids to proteins of a molecular weight of 100,00 or more. While the major neuroactive ions and amino acids/amines were isolated and chemically defined by the 1960s, the identification and structural elucidation of the neuropeptides date to as recently as 1969 with the elucidation of

the chemical structure of TRH (throtropin-releasing hormone). Since then, numerous neuropeptides have been identified.

Decades ago it was thought that the brain peptidergic substances were restricted to the hypothalamic-pituitary axis, where hypothalamic releasing factors specifically controlled release of pituitary hormones. Throughout the 1970s, the development of radioimmunological assays and immunohistochemistry led to the demonstration of numerous different peptides in neuronal networks throughout the brain. About this time, it was suggested that peptides represented a whole new generation of neurotransmitters, and that study of those substances could provide important clues to the mechanisms of neuronal communication (Bloom, 1973; Iversen, 1974). Since then, the number of peptides demonstrated in nervous tissue has grown rapidly, a process which still continues. In parallel, considerable efforts have been aimed at understanding the behavioral function of neuropeptides, within domains of study as diverse as learning and memory, response to stress and pain, ingestive behavior, and motivation.

Researchers were originally quite unwilling to accept such large, complex molecules as peptides, which were presumably synthesized at ribosomes within the neuronal cell body, as synaptic transmitters. We now know that many of these substances have a specific synaptic function; moreover, recent developments in molecular genetic studies suggest that specific mechanisms at the transcriptional level may play an important role in information encoding and ultimately in global

behavioral functions (Bloom, 1979; Schmitt, 1984). For example, opioid peptides are derived from three distinct prohormones, proopiomelanocortin, proenkephalin, and prodynorphin. In some cases proenkephalin- and prodynorphin-derived peptides may be co-localized, since they appear to have the same distribution. The substantia nigra and VTA (ventral tegmental area) both contain dynorphin and enkephalin. In the study by Bannon, amounts of these two peptides were measured in the VTA after footshock stress (Bannon *et al.*, 1986). Footshock markedly depleted dynorphin but left enkephalin levels unchanged. In support of differential actions of the opioid peptides, the work of Kanamatsu showed that dynorphin and met-enkephalin are altered differentially by repeated electroconvulsive shock treatment (Kanamatsu *et al.*, 1986). Evidence such as these had important consequences for concepts about the neural coding of behavior. The information coding potential inherent in these molecules and their associated genetic operation is great. The existence of multiple forms of the same peptide or similar peptides suggests that peptides may play a fundamental role in the elaboration of complex, adaptive behavior.

The discovery of a wide range of biologically active peptides (adrenocorticortropin, α-endorphin, β-endorphin, bradykinin, dynorphin, gastrin, growth hormone, insulin, kyotorphin, leu-enkephalin, met-enkephalin, neurotensin, secretin, substance P, TRH, vasopressin, etc.) in the nervous system and growing knowledge of their characteristics has contributed to new theories of information transfer and chemical integrative process in the brain (Bloom, 1979; Schmitt, 1984).

Considering the number of different amino acid combinations, different chain lengths, and multiple forms of related peptides, the actual number of peptides in the brain could be very large indeed. It is a truly formidable and baffling task for the behavioral neuroscientist to incorporate this geometrically expanding body of knowledge into working hypotheses about behavior. Thus the study and understanding of mammalian behavior, which are never simple, become even more complex. One can hope nevertheless that in the future research an integration of refined behavioral techniques and paradigms with knowledge of the basic neurobiology of peptides may contribute at least partially to this understanding.

Neuropeptides as Potential Neuropharmaceuticals

Disorders of the brain are surprisingly common. These disorders include headache, alcohol abuse, anxiety/phobia, sleep disorders, depression/mania, drug abuse, obsessive-compulsive disorder, Alzheimer's disease, stroke, epilepsy, and Parkinson's disease. The drugs currently used for disorders of the brain such as codeine (headache), diazepam (anxiety), amitryptiline (depression), and phenytoin (epilepsy) are all lipid-soluble pharmaceuticals that readily cross BBB following oral administration. These drugs were discovered with the traditional "trial-and-error" method of drug discovery wherein up to 10,000 different compounds were screened with *in vivo* bioassays (Waldrop, 1990). This approach excluded drugs that may have interacted with particular receptors in the brain but were drugs that had poor

BBB transport properties owing to the low lipid solubility of the compound, such as neuropeptides.

The multiplicity of biological actions of peptides in the brain suggests that these agents may be utilized as neuropharmaceuticals in the treatment of a variety of disorders of the brain and spinal cord. However, as with any potential neuropharmaceutical, peptides must be able to undergo transport into the brain from blood. Neuropeptide investigation in the mid and late 1970s underscored the importance of a through understanding of the mechanisms by which peptides are transported between blood and the brain.

Shortly after the discovery of peptides such as TRH or β-endorphin, these agents were infused intravenously into humans for the treatment of psychiatric disorders such as schizophrenia or depression (Barchas & Elliott, 1986). No consistent effects on the brain were found with these agents in humans. In the case of β-endorphins, the peptide had profound effects on the central nervous system in rats following the injection of the peptide directly into the ventricular compartment. However, no effects were found after the systemic administration of very large doses of β-endorphin into rats or humans. The failure of any central actions of β-endorphin following peripheral administration is due to the presence of a barrier between blood and the brain, the blood-brain barrier (BBB).

The lack of transport of most neuroactive peptides through the BBB parallels the absence of transport of the neuroactive amino acids or monoamines such as

dopamine. In the 1960s, the neutral amino acid L-dihydroxyphenylalanine (L-dopa) was developed for the treatment of Parkinson's disease, a degenerative condition of the brain wherein the caudate putamen region of the brain forms inadequate amounts of dopamine. L-dopa can be transported into the brain via the BBB neutral amino acid transport system and, subsequent to this transport, is converted to dopamine by brain aromatic amino acid decarboxylase (Wade & Katzman, 1975). Unfortunately, peripheral tissues are also endowed with aromatic amino acid decarboxylase, and this results in the peripheral conversion of L-dopa to dopamine.

The use of L-dopa in Parkinson's disease is the paradigm of single neurotransmitter repletion therapy of neurologic disease and illustrates at least two important principles applicable to the use of neuropeptides as neuropharmaceuticals. The first principle is that neurotransmitter or neuromodulator requires a drug delivery system to cross the BBB. The second principle is the need to slow the rapid enzymatic inactivation of the neuropharmaceuticals so that adequate amounts can reach appropriate sites within the brain.

In this dissertation, I will apply these principles to the use of kyotorphin, an endogenous opioid neuropeptide, as a potential neuropharmaceutical in the treatment of pain.

The Opioids

The term *opiate* was once used to designate drugs derived from opium -- morphine, codeine, and the many semisynthetic congeners of morphine. Soon after the development of totally synthetic entities with morphine like actions, the word *opioid* was introduced to refer in a generic sense to all drugs, natural and synthetic, with morphine-like actions.

There are now many compounds that produce analgesia and other effects similar to those produced by morphine (Fig. 1). Some of these may have some special properties, but none has proven to be clinically superior in relieving pain.

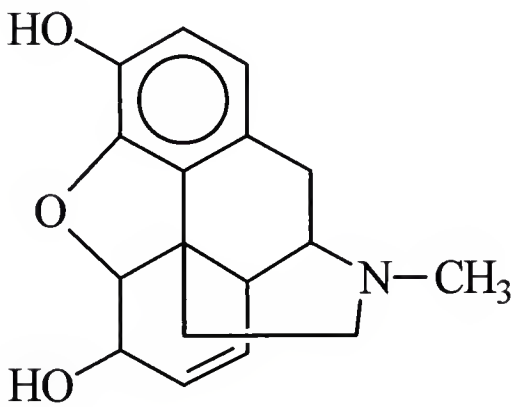


Figure 1. The Structure of Morphine

Morphine remains the standard against which new analgesics are measured. Because the laboratory synthesis of morphine is difficult, the drug is still obtained from opium or extracted from poppy straw.

In man, morphine-like drugs produce analgesia, drowsiness, changes in mood, and mental clouding, etc. A significant feature of the analgesia is that it occurs without loss of consciousness. When therapeutic doses of morphine are given to patients with pain, they report that the pain is less intense, less discomforting, or entirely gone. The relief of pain by morphine-like opioids is relatively selective, in that other sensory modalities (touch, vibration, vision, hearing, *etc.*) are not affected. Continuous dull pain is relieved more effectively than sharp intermittent pain, but with sufficient amounts of morphine, it is possible to relieve the severe pain such as those associated with renal or biliary colic.

Opioid-induced analgesia is due to actions at several sites within the CNS and involves several systems of neurotransmitters. Although opioids do not alter the threshold or responsitivity of afferent nerve endings to noxious stimulation or impair the conduction of the nerve impulse along peripheral nerves, they may decrease conduction of impulses of primary afferent fibers when they enter the spinal cord and decrease activity in other sensory endings. There are opioid binding sites (μ receptors) on the terminal axons of primary afferents within laminae I and II (substantia gelatinosa) of the spinal cord and in the spinal nucleus of the terminal nerve. Morphine-like drugs acting at this site are thought to decrease the release of

neurotransmitters, such as substance P, that mediate transmission of pain impulses. Enkephalinergic nerve fibers in the dorsal horn of the spinal cord, which appear to come from interneurons, are usually inhibitory to dendrites and some of nerves whose cell bodies may be in deeper laminae (IV and V). It can be inferred that in the spinal cord, separate μ and δ receptors participate in inhibiting transmission of pain impulses.

Other than analgesia, morphine can produce nausea, vomiting, feeling of drowsiness, inability to concentrate, difficulty in mentation, apathy, lessened physical activity, reduced visual acuity, body warmth, relief of stress, and euphoria (Duggan & North, 1983; Martin, 1983)

The History of Opioid Peptides and Their Receptors

Morphine was the first natural opioid to be identified and characterized. This alkaloid was isolated as one of the analgesic components of opium. The question of why a plant alkaloid should bind to stereospecific receptors on neuronal membranes and display such dramatic effects on the mammalian nervous system was answered by an extensive search for an endogenous opioid ligand to the mammalian nervous system. Initial studies were aimed at the isolation of alkaloids, but it soon became apparent from many experiments that peptides may be endogenous ligands for the opioid receptors.

The Opioid Peptides

The early evolution of concepts of endogenous opioids and multiple opioid receptors had its inception in a concerted program to develop safe, non-addicting substitutes for opioids. Although the search for safer and less-abusable analgesics has not been entirely successful, ideas concerning multiple opioid receptors and endogenous opioid transmitters were evolved during the process.

In the early 1970s, two pentapeptides with opioid-like activity were extracted from porcine brain and characterized (Hughes *et al.*, 1975). These two peptides differed only in the C-terminal amino acid and were named methionine enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu). The isolation of the other two families of opioid peptides, the β-endorphins and the dynorphins, was also accomplished in 1975 (Bradbury *et al.*, 1975; Cox *et al.*, 1975).

Each family of opioid peptides is derived from a genetically distinct precursor polypeptide and has a characteristic anatomical distribution. These precursors are now commonly designated as proenkephalin (also proenkephalin A), pro-opiomelanocortin (POMC), and prodynorphin (also proenkephalin B). Each of these precursors contains a number of biologically active peptides, both opioid and nonopioid, that have been detected in blood and various tissues. Proteolytic processing of the precursors generates the active peptides which are expressed via peptide receptor systems at the target cell -- the opioid peptide receptors.

In addition to products belonging to these major families, other opioid peptides have been identified in the brain (kyotorphin) and frog skin (dermorphin).

The Opioid Peptide Receptors

When considering the most basic requirements for a neurotransmitter system, one must include certain specific receptors as well as ligands. In fact, in the case of the endogenous opioids the existence of opioid receptors preceded the discovery of the opioid peptide ligands. It has been possible to study opioid receptors by using receptor binding assays. In this technique the interaction of a radio-labeled opioid ligand with the receptor may be monitored *in vitro*. Extensive characterization of the opioid receptor has taken place using these biochemical techniques and some important conclusions may be summarized as follows:

1) The receptors appear to be proteins (Miller & Dawson, 1980; Pasternak & Snyder, 1976; Zukin & Zukin, 1984). High affinity, saturable, stereo-specific binding sites may be labeled by using a variety of ligands, including opioid agonists opioid antagonists, and opioid peptides.

2) Multiple opioid receptors exist. Martin originally suggested that three different categories of receptor existed based on the analysis of the effects of a wide range of narcotics in the chronic spinal dog preparation. These categories were designated μ (mu), κ (kappa), and σ (sigma) (Gilbert & Martin, 1976; Martin *et al.*, 1974, 1976). In addition to these categories, Kosterlitz and colleagues suggested a

further category designated δ (delta) on the basis of differential effects of opioid peptides and narcotic drugs on the guinea pig ileum and mouse vas deferens bioassays (Kosterlitz *et al.*, 1979). The psychotropic actions of opioids and opioid peptides are mediated by interaction with μ, δ, and κ opioid receptors and the related σ receptor. The μ receptor is operationally defined as the high-affinity site at which morphine-like opioids produce analgesia and other classical opioid effects. The δ receptor is operationally defined as the receptor that is found in peripheral tissues such as the mouse vas deferens as well as in the CNS (Chang *et al.*, 1979), and that exhibits a higher affinity for naturally occurring enkephalins than for morphine. The κ receptor is the receptor at which ketocyclazocine-like opioids produce analgesia as well as their unique ataxic and sedative effects (Gilbert & Martin, 1976; Martin *et al.*, 1976). It has been defined as a receptor highly selective for dynorphin -- a 17 amino acid opioid peptide (Chavkin *et al.*, 1982). Actions at all three of these sites are reversible by a specific opioid antagonist -- naloxone, with decreasing sensitivity going from μ to δ to κ receptors. The σ receptor was proposed to be the site at which the psychotomimetic and stimulatory effects of *N*-allylnormetazocine, cyclazocine, and related opioid are mediated (Martin *et al.*, 1976; Zukin & Zukin, 1981).

In summary, analgesia is associated with μ and κ receptors, while dysphoria or psychotomimetic effects is ascribed to σ receptors. δ receptor is involved in altercation of affective behavior.

Opioid Actions Related to the Central Nervous System (CNS)

The distribution of opioid receptors in the CNS is revealed in detail by the autoradiographic method which generally agrees well with the biochemical binding studies. Using [^{3}H]diprenorphine, an antagonist with high affinity with opioid binding sites, Kuhar's group developed the first autoradiographic technique used to localize opioid receptors at the light microscopic level. In 1977, Atweh and Kuhar published a detailed description of the distribution of [^{3}H]diprenorphine binding throughout the central nervous system (Atweh & Kuhar, 1977 a, b, c), providing an excellent correlation between regions capable of mediating the various opioid actions (analgesia, autonomic reflexes, endocrine effects, behavioral and mood effects, and motor rigidity) and those containing receptors.

Analgesia

Opioids and the opioid peptides have the unique ability to selectively relieve the subjective component of pain without affecting primary sensory modalities, such as touch, vibration, vision, and hearing. Opioids can modulate pain sensation at several levels of the CNS. Most experimental work has focused on the spinal cord,

specifically, laminae I and II and the Periaqueductal gray (PAG). The physiology of this system has been extensively studied (Basbaum, 1984).

Respiratory Depression

Classical μ and δ opioids and the opioid peptides consistently depress respiration in all species that have been tested. These actions appear to involve both decreased responsiveness of the CNS centers to CO_2 and a decrease in the CNS respiratory frequency controller (Eckenhoff & Oech, 1960; McQueen, 1983).

Other Opioid Actions Mediated by the CNS.

The other opioid actions mediated by the CNS, such as autonomic reflexes, endocrine effects, behavioral and mood effects, and motor rigidity, are summarized in Table 1.

Opioid Actions Related to the Peripheral Tissues

Gastrointestinal Tract

Bioassays of intestinal extracts confirmed that the presence of enkephalin-like peptides in intestinal extracts (Hughes *et al.*, 1977). In all mammalian studied, neurons in the myenteric plexus staining with anti-enkephalin antisera have been found. The relative number of these enkephalin-containing cells appears to vary both with regard to location along the gastrointestinal tract from esophagus to colon and among species. In general, fibers from the enkephalin-containing cells run from and between myenteric plexus ganglia and distribute extensively through the circular

Table 1. Correlation of Opioid Action with Receptor Localization in the CNS

Opioid Action	Receptor Localization
Analgesia	
Spinal, body	Lamine I and II of spinal cord
Trigeminal, Face	Substantia gelatinosa of trigeminal nerve
Superaspinal	Periaqueductal gray, medial thalamic nuclei, intralamellar thalamic nuclei
Autonomic Reflexes	
Cough suppression, orthostatic hypotension, inhibition of gastric secretion	N. tratus solitarius, commissuralis, ambiguus, and locus ceruleus
Respiratory depression	N. tractus solitarius, parabrachial nuclei
Nausea and vomiting	Area postrema
Meiosis	Superior colliculus, pretectal nuclei
Endocrine Effects	
Inhibition of vasopresin release	Posterior pituitary
Hormonal effects	Hypothalamic nuclei, accessory optic system amygdala
Behavioral and Mood Effects	Amygdala, N. stria terminalis, hippocampus cortex, nedial thalamic nuclei, N. accumbens, basal ganglia
Motor Rigidity	Striatum

and longitudinal muscle layers. Some fibers also innervate the submucus plexus and the mucosa, but enkephalin cell bodies are rare or absent in the submucus plexus. Opioid receptors in the gastrointestinal tract were found in intestine, esophageal sphincter, stomach circular muscle, duodenum, ileum, and rectum among species (Elde *et al.*, 1976; Linnoila *et al.*, 1978; Jessen *et al.*, 1980; Schultzberg *et al.*, 1980; Uddman *et al.*, 1980).

Opioids can exert several different actions on gastrointestinal motility, such as reduced gastrointestinal propulsion and secretion. The overall result of these actions is a decline in propulsive activity, resulting in a delay in the passage of gastrointestinal contents. The reduction in propulsion of gastrointestinal contents allows longer time for fluid reabsorption. A reduction in fluid secretion also contributes to the production of dry, compacted feces (Powell, 1981). It is possible that opioids can act at both central and peripheral sites to alter gastrointestinal function after systemic administration (Schulz *et al.*, 1979; Manara & Bianchetti, 1985). Those actions contributed to the antidiarrheal action of opioids.

Adrenal Gland

The presence of enkephalins in adrenal medulla has been revealed by Schutlzberg (Schutlzberg *et al.*, 1978). Subsequently, adrenal medullary tissue has been used as a major source of enkephalin precursors (Lewis *et al.*, 1980) and of

proenkephalin mRNA (Noda *et al.*, 1982; Comb *et al.*, 1982). Enkephalins and the larger molecular weight peptides are released from adrenal medulla together with catecholamine in response to potassium perfusion of the gland or stimulation of the splanchnic nerve (Viveros *et al.*, 1979; Chaminade *et al.*, 1984).

The Sympathetic Nervous System

The presence of enkephalin-like material in sympathetic ganglia has been demonstrated and, as in adrenal gland, has been shown to consist of both the pentapeptide enkephalins and higher molecular weight enkephalin containing peptides (Di Giulio *et al.*, 1978). The immunocytochemical localization of enkephalin peptides in sympathetic ganglia of guinea pig, rat, and human has also been described (Schutlzberg *et al.*, 1979; Hervonen *et al.*, 1981).

Blood Vessels

The ability of enkephalins to depress vasoconstrictor response to sympathetic nerve stimulation in the rabbit isolated ear artery was first noted by Knoll (Knoll, 1976) and was confirmed by other groups soon after (Ronal *et al.*, 1982; Illes *et al.*, 1983). The locus of action has been shown to be at the presynaptic nerve terminal. In this tissue, morphine is less effective than the opioid peptides.

Enkephalins can also exert non-opioid effects on blood vessels. Pulmonary vasoconstriction induced by leu-enkephalin is not reversed by naloxone or

diprenorphine. Other enkephalins, including met-enkephalin and DADLE (D-Ala^2-D-Leu^5-enkephalin), do not induce the same vasoconstrictor effect (Crooks *et al.*, 1984). Thus, a non-opioid mechanism is assumed to be responsible for this effect.

The Heart

Endogenous opioids have been reported to be present in guinea pig and rat heart (Hughes *et al.*, 1977; Spampinato & Goldstein, 1983; Lang *et al.*, 1983). Proenkephalin mRNA has also been found in rat heart (Howells *et al.*, 1985). They found that the heart contained larger amounts of enkephalin mRNA than any other tissue and 95% of the mRNA was found in the ventricles which contain very low concentrations of enkephalin, relative to both atria and to other tissue like brain. Pharmacological studies suggested that the opioid binding site might be located on the autonomic innervation of the heart (Saunders & Thornhill, 1985).

Intravenous administration of morphine and some opioid peptides in rat has been shown to produce a transient bradycardia that is blocked by atropine or vagotomy (Fennessy & Rattray, 1971; Wei *et al.*, 1980). The effect is peripherally mediated, since it is antagonized by the quaternary antagonist *N*-methylnaloxonium (Kiang *et al.*, 1983).

Sensory Nerves

Endogenous enkephalins have been found in tooth pulp (Kudo *et al.*, 1983) and in the carotid body (Wharton *et al.*, 1980). In the spinal cord, there are high concentrations of enkephalins and dynorphins in the substantia gelatinosa (Hunt *et al.*, 1980; Vincent *et al.*, 1982), where collateral's of primary sensory neuron's synapse with regulatory interneurons. Thus, endogenous opioids appear to be strategically located for the regulation of transmission from the primary afferent neuron to the central nervous system. The opioids might reduce the output of transmitter from primary afferent neurons, reducing their ability to activate secondary sensory neurons (Werz & Macdonald, 1982, 1984).

Reproductive Tract Tissues

All three classes of endogenous opioid peptides have been found in the tissues of the reproductive tract (Sharp *et al.*, 1980; Margioris *et al.*, 1983; Lim *et al.*, 1983; Pintar *et al.*, 1984). The functions of reproductive tract endogenous opioids are still unclear.

In summary, the localization of opioid receptors and enkephalin-containing neurons in the thalamus, Periaqueductal gray matter, and substantia gelatinosa of the spinal cord suggests a role for endogenous opioid peptides in pain modulation at these three levels (Hokfelt *et al.*, 1977). A major end point of all studies of opioid

and opioid peptide action is the development of new compounds with clinical utility. To date, opioids have been used primarily as analgesics and antidirrheals. The importance of opioid mechanisms in a wide range of neuropharmacological actions, however, suggests that these agents may have a much wider use. The development of an agent which is capable of producing desired actions with fewer side-effects is desired.

The Enkephalins

The enkephalins were first isolated and sequenced by Hughes (Hughes *et al.*, 1975) from a mixture of two pentapeptides present in the extracts of porcine brain which differed only by possessing either a Met or Leu residue at their C-terminal. The two peptides were named met-enkephalin (NH_2-Tyr-Gly-Gly-Phe-Met-OH) and leu-enkephalin (NH_2-Tyr-Gly-Gly-Phe-Phe-OH). Later the same two compounds were obtained from bovine brain (Simantov & Snyder, 1976).

Under suitable assay conditions, the enkephalins have many of the biological characteristics of the alkaloid opioids; however, their susceptibility to enzymatic break down and short duration of action made their study difficult. Met- and leu-enkephalin both bind to opioid receptors on brain cell membrane preparations with comparable affinities to morphine when experiments are performed at 0°C or in the presence of proteolytic enzyme inhibitors (Chang *et al.*, 1976). In systems where enzymatic activity is higher, such as vas deferens and ileum assays, the peptides are

considerably less active than morphine. For induction of analgesia, very high doses of met-enkephalin must be directly introduced into the brain and leu-enkephalin is even less active (Beluzzi *et al.*, 1976; Buescher *et al.*, 1976).

Early interest in the enkephalins centered on whether these endogenous substances would induce tolerance and withdrawal symptoms in a similar way to the morphinoids. This was unfortunately the case in a number of paradigms. For instances, several investigators have detected cross-tolerance between morphine and enkephalins and some of their analogs (Waterfield *et al.*, 1976; Bhargava, 1978).

Apart from analgesic activity, both met- and leu-enkephalin have effects on the secretion of several pituitary hormones. Principal among these are stimulatory actions on growth hormone and prolactin release which appear to be mediated by hypothalamic growth hormone-releasing hormone and possibly catecholamines, respectively (Dupont *et al.*, 1979; Meites *et al.*, 1979; Cusan *et al.*, 1977). Both enkephalins were also found to significantly decrease serum LH (luteinizing hormone; Meites *et al.*, 1979). Because of the multi-type opioid receptors, it is not surprising that the enkephalins and their analogs exhibit behavioral properties which cannot be duplicated by using the morphinoids.

Kyotorphin (KTP)

Kyotorphin (KTP, L-Tyrosyl-L-Arginine) is an endogenous neuropeptide that exhibits analgesic action by mediation of the release of endogenous enkephalins from

nerve terminals inside the brain. The name of kyotorphin means an endorphin-like substance which was discovered in Kyoto, Japan. The analgesic potency of kyotorphin is 4.2 times that of met-enkephalin (Takagi *et al.*, 1980).

Kyotorphin administered intracisternally elicits a naloxone-reversible antinociception. This peptide does not bind to μ-, δ-, and κ-sub-types of opioid receptors, but does induce an enhancement of met-enkephalin release in brain and spinal cord slices (Takagi *et al.*, 1979). Thus, kyotorphin-induced analgesia may be attributed to the enhanced release of met-enkephalin in the brain stem and spinal dorsal horn involved in pain transmission.

Its analgesic effects may also result from its inhibition of the enzymatic hydrolysis of other endogenous opioid peptides (Takagi *et al.*, 1979). The selective inhibition by kyotorphin and neo-kyotorphin (Thr-Ser-Lys-Tyr-Arg) on enkephalin-degrading enzymes suggests that kyotorphin might protect the released met-enkephalin. Thus, kyotorphin may not only induce the release of met-enkephalin, but also stabilize the released neuropeptide (Hazato *et al.*, 1986). Satoh has suggested that the analgesic actions of kyotorphin and D-kyotorphin result from two different mechanisms: 1) enkephalin releasing mechanism in the PAG and spinal dorsal horn, and 2) a mechanism without involvement of enkephalin-releasing actions in the NRPG (nucleus reticularis paragigantocellularis) (Satoh *et al.*, 1985).

Kyotorphin is formed through two discrete pathways; one is formation from L-arginine and L-tyrosine by a specific synthetase (Ueda *et al.*, 1987); the other

pathway is formation through the processing of precursor proteins by a neutral protease (Yoshihara *et al.*, 1988).

It was reported that kyotorphin is lower in concentration in patients with persistent pain, which suggests that kyotorphin acts as a putative neuromediator and/or an endogenous pain modulator in the human brain (Nishimura, 1991). As with stimulation-produced analgesia, several studies suggest that Chinese traditional acupuncture analgesia may be mediated, at least in part, by endogenous opioid peptides (Mayer *et al.*, 1977).

Analgesia, opioid receptor binding, and neurochemical effects of kyotorphin were studied in rat. It was found that while kyotorphin, *in vivo*, causes naloxone reversible analgesia, and affects dopamine metabolism and acetylcholine turnover in the same manner as does morphine and other opioid agents, the dipeptide does not bind to μ-, δ-, and κ-opioid receptors *in vitro* (Rackham *et al.*, 1982). The above data support the concept that there is an indirect action of kyotorphin on opioid receptors. Stone also concluded that the naloxone-reversible analgesic effects of kyotorphin and D-phenylalanine may be mediated indirectly, rather than through an activation of opioid receptors (Stone, 1983).

Kyotorphin may also play a neurotransmitter/neuromodulator role in the brain or may increase transmitter release from preganglionic nerve terminals inside CNS (Ueda *et al.*, 1982; Hirai *et al.*, 1985). It was reported that specific high and low affinity kyotorphin receptors exist in the rat brain and that the kyotorphin

receptor is functionally coupled to stimulation of phospholipase C through Gi (Ueda *et al.*, 1989).

Kyotorphin is rapidly hydrolyzed in the brain (Ueda *et al.*, 1985). Specific kyotorphin-hydrolyzing enzymes -- KTPase I and II have been identified recently (Akasaki *et al.*, 1991). Kyotorphin is also easily degraded by other unspecific hydrolyzing enzymes, such as enkephalin-degrading aminopeptidase as well as other peptidases (Akasaki & Tsuji, 1991).

Many kyotorphin analogs has been synthesized and tested. Table 2 shows some of the kyotorphin analogs that have shown analgesic activities (Rolka *et al.*, 1983; Carcia-Lopez *et al.*, 1987; Carcia-Lopez *et al.*, 1988).

Table 2. Some Kyotorphin Analogs

Names of the Compounds	
D-Tyr-Arg	Arg-Tyr
Tyr-D-Arg	cyclo(Tyr-Arg)
Tyr-Lys	cyclo(Trp-Arg)
D-Tyr-Lys	Lys-Trp(NPS)
Tyr-Orn	Lys-Trp(NPS)-OMe
D-Tyr-Orn	Arg-Trp(NPS)-OMe

The structure-activity relationship of a dipeptide with met-enkephalin-releasing action includes an aromatic acid with a basic nitrogen on the ring (Tyr or Trp) and a basic amino acid (Arg or Lys or Orn). The basic side-chain amine group of the basic amino acid, which is positively charged *in vivo*, is critical to the activities, although the side-chain length is not a critical factor.

Tyrosyl-lysine (KAYK) is a kyotorphin analog which exhibits the same analgesic effect as kyotorphin when administered i.c., 1 mg/kg, in rats (Rolka *et al.*, 1983).

Peptide and Protein Drug Delivery

The search for effective and safe drugs continues to be a major effort, involving the pharmaceutical industry, universities, and government. The complexities of discovering and testing new drugs have become enormous as a result of the many aspects of safety, efficacy, and economics that determine acceptability of a drug. The concept of drug carriers or drug delivery systems has been embraced with great enthusiasm by many as the solution. For example, toxicity would be reduced by delivering a drug to its target in higher concentrations, reducing harmful systemic effects by decreasing the quantity of administered drug needed to produce a desired effect. Toxicity could also be reduced by administering the drug in a non-toxic form that is activated only at the site of action. More broadly, drug delivery systems could modify various parameters, such as pharmacokinetics, so as to permit

the effective use of drugs that by themselves are not useful and efficacious. Finally, the rational and scientific design of drugs could be greatly facilitated by their development in conjunction with an appropriate carrier system. In reality, drug carriers became entrapped in many of the same problems that they were intended to solve, such as potential toxicity, efficacy, and so forth. Nevertheless, their potential remains bright. The difficulty of finding new pharmacological therapies for diseases not already amenable to existing drugs is so great that if drug carriers were to lead to the successful treatment of only a single important disease, that success alone would easily justify the research time and funds that have been expended on the development of drug carriers. It seems likely that drug carriers will be an increasing important aspect of pharmacology and therapeutics in the future.

Hormones, serum proteins, and enzymes have been used as drugs ever since the commercial introduction of insulin, thyroid hormone, and factor VIII from 1920 through 1940. Molecular biology has now given us the tools to expand the range of peptide- and protein-based drugs to combat poorly controlled diseases. While there has been rapid progress in molecular biology, this has not been matched by the progress in the formulation and development of peptide and protein delivery systems. This is due, in part, to the lack of appreciation for the unique demands imposed by the physicochemical and biological properties of peptides and protein drugs on route of delivery as well as on delivery system design and formulation. These properties include molecular size, short plasma half-life, requirement for

specialized mechanisms for transport across biological membranes, susceptibility to breakdown in both physical and biological environments, tendency to under go self-association, complex feedback control mechanisms, and particular dose-response relationships.

Neuropeptide-Degrading Enzymes

Termination of the biological actions of neuropeptides is very important to organisms and appears to occur predominantly through metabolism by cell-surface membrane peptidases (Lynch & Snyder, 1986). One of the enzymes that has been most investigated is a plasma membrane metalloendopeptidase that participates in the metabolism of the enkephalins. When studied in the brain, the enzyme has hence been termed "enkephalinase", although the enzyme is widely distributed and probably participates in the metabolism of other neuropeptides (Matsas *et al.*, 1983). The nomenclature "endopeptidase-24.11" is now regarded as a more appropriate name (Hersh, 1986). A number of other membrane exopeptidases and endopeptidases hydrolyzing neuropeptides have been isolated and characterized, including peptidyl dipeptidase A (angiotensin-converting enzyme; ACE) and aminopeptidase N (AP). Some of the peptidases presented in the CNS are listed in Table 3. There are many other peptidases which might present in the CNS, such as carboxypeptidase P (EC3.4.17.-), carboxypeptidase N (EC 3.4.17.3), aminopeptidase A (EC 3.4.11.7), aminopeptidase P (EC 3.4.11.9), and aminopeptidase W (Turner *et al.*, 1989).

Table 3. Some Peptidases Present in the CNS

Enzyme	Active site	Specificity	Specific inhibitors
Endopeptidase-24.11 EC 3.4.24.11	Zn^{2+}	-O-O-X-O ↑ hydrophobic	Phosphoramidon Thiorphan
Angiotensin-Converting Enzymes EC 3.4.15.1	Zn^{2+}	-O-O-X-X- ↑ non-specific	Captopril Enalaprilat Lisnopril
Endopeptidase-24.15 EC 3.4.24.15	Zn^{2+}?	-O-O-X-O-O- ↑ hydrophobic	N-(1(R,S)-carboxy-2-phenylethyl)-Ala-Ala-Phe-pAB
Aminopeptidase N EC 3.4.11.2	Zn^{2+}	X-O-O-O- ↑ NH_2- terminal	Amastatin Bestatin Actinonin
Dipeptidyl peptidase IV EC 3.4.14.5	serine	-O-X-O-O- ↑ Pro or Ala	Diisopropyl-fluorophosphate (Dip-F)
Post-proline cleaving enzyme EC 3.4.21.26	serine	-O-Pro-O-O- ↑	Dip-F Z-Pro-prolinal
Pyroglutamyl peptidase II EC 3.4.19.-	metallo-peptidase	Glp-His-Pro-NH_2 ↑	?
Microsomal dipeptidase EC 3.4.13.11	Zn^{2+}	-O-O- ↑ (non-specific)	Cilastatin

Endopeptidase-24.11

Endopeptidase-24.11 (enkephalinase; neutral metallo-endopeptidase; EC 3.4.24.11) is a widely distributed enzyme. In the nervous system, the enzyme has been implicated in the metabolism of the enkephalins which are hydrolyzed at the Gly^3-Phe^4 bond with the release of Tyr-Gly-Gly. The broad substrate specificity of endopeptidase-24.11, however, suggests that it may also participate in the degradation of tachykinins (e.g. substance P), neurotensin, cholecystokinin, kyotorphin, and other neuropeptides.

Endopeptidase-24.11 is an integral membrane glycoprotein of *M*r 85,000 - 95,000, depending on the tissue, and in most species, exists as a dimer in the plasma membrane. One Zn^{2+} is bound at the active site of the enzyme which is essential for its catalytic activity. Endopeptidase-24.11 hydrolyses peptide bond involving the amino groups of hydrophobic residues, (X-Y- where Y is Phe, Leu, Ile, Ala, Val, Tyr, or Trp). Several specific inhibitors of the enzyme exist, of which the most widely used is Phosphoramidon Thiorphan (Turner *et al.*, 1985).

Peptidyl Dipeptidase A

Peptidyl Dipeptidase A (angiotensin-converting enzyme; ACE; kininase II; EC 3.4.15.1) is widely distributed throughout the mammalian body, with the lung and kidney having the highest concentration of enzyme per gram of tissue. At the vascular surface of the lung, the enzyme converts angiotensin I to angiotensin II and

also inactivates bradykinin (Soffer, 1976). In the brain, the striatum, substantia nigra and choroid plexus are enriched in ACE. The endogenous substrates for ACE in the brain are unclear, although it has been implicated in the metabolism of C-terminally extended enkephalin peptides, neurotensin, and substance P.

The enzyme is a glycoprotein of *M*r about 13,000. It has one Zn^{2+} bound at its active site which is essential for its catalytic activity. Originally, ACE was classified as a peptidyl dipeptidase. Recently, certain endopeptidase and tripeptidase activity have been attributed to the enzyme.

Aminopeptidase N

Aminopeptidase N (AP-N, aminopeptidase M; alanyl aminopeptidase; EC 3.4.11.2) is an integral membrane glycoprotein of *M*r ~ 160,000. The enzyme has a very broad distribution. It is highly concentrated in the vasculature in the CNS. The active site of AN-P contains one Zn^{2+}.

Aminopeptidase N has a broad specificity, releasing N-terminal amino acids from unblocked di, tri, and oligopeptides. When the N-terminal amino acid is Ala, hydrolysis is most rapid. Aminopeptidase N is the major aminopeptidase releasing Tyr from met- and leu-enkephalin (Matsas *et al.*, 1985), and may function to degrade other neuropeptides with free N-terminus. Hydrolysis is more rapid with extended peptides than with dipeptides when chain length is considered.

Dipeptidyl Peptidase IV

Dipeptidyl Peptidase IV (DPP-IV; EC 3.4.14.5) is a plasma membrane peptidase widely distributed in mammalian tissues. It has been reported to present in the brain where it appears to have a diffuse distribution. In kidney, the enzyme exists as a dimer with subunit *M*r of ~ 130,000. DPP-IV is a serine peptidase and is therefore inhibited by diisopropylflurophosphate (Kenny *et al.*, 1976).

The specificity of the enzyme is to remove dipeptides from the N-terminus of unblocked oligopeptides as follows: W-X-Y- → W-X + Y-, when X= Pro or Ala. The rates with Ala are about one-fifth those with Pro.

Post-Proline Cleaving Enzyme

Post-Proline Cleaving Enzyme (post-proline endopeptidase; PPCE; Prolyl endopeptidase; EC 3.4.21.26) is a dimeric enzyme of subunit *M*r 62,000 ~ 77,000. The enzyme is widely distributed in mammalian tissues, with brain being one of the richest sources. The enzyme cleaves peptide bonds on the C-terminal side of proline as follows: -V-Pro-X-Y- → -V-Pro + X-Y-. PPCE is a serine peptidase sensitive to inhibition by diisopropylflurophosphate (Wilk, 1983).

Microsomal Dipeptidase

Microsomal Dipeptidase (renal dipeptidase; dehydropeptidase I; EC 3.4.13.11) is an integral membrane glycol protein of *M*r ~ 47,000 (Hooper *et al.*,

1987). The enzyme has been identified in kidney, pancreas, liver, spleen, lung, and brain. The active site of microsomal dipeptidase contains one Zn^{2+}.

Microsomal dipeptidase has a broad specificity towards dipeptides, including dipeptides in which the C-terminal residue has the D-configuration. Longer peptides are not substrates for the enzyme. In the nervous system the enzyme may serve to hydrolyze dipeptide neurotransmitters (e.g., kyotorphin or glycyl-glutamine). Dipeptides can also arise as secondary metabolites from the hydrolysis of other neuropeptides by membrane peptidases.

The Enzymatic Barrier For Peptide and Protein Drug Delivery

Potential peptide and protein drugs are subject to degradation by numerous enzymes or enzyme systems throughout the body. This degradation can come in two forms: 1) hydrolytic cleavage of peptide bonds by proteases, such as enkephalinases, and 2) chemical modification of the peptides or proteins, such as oxidation and phosphorylation. Hydrolysis is by far the more common. Therefore, a major challenge in peptide and protein drug delivery is to overcome the enzymatic barrier that limits the amount of peptide and protein drugs from reaching their targets. Degradation usually begins at the site of administration and can be extensive. Even when the subcutaneous or intramuscular route is used, less than complete bioavailability is often observed. For instance, the subcutaneous or intramuscular bioavailability of TRH, a tripeptide, in mice is only 67.5% and 31.1%, respectively

(Redding & Schally, 1972). The enzymatic barrier has three essential features, as follows.

First, since proteases and other proteolytic enzymes are ubiquitous, peptides and proteins are usually susceptible to degradation in multiple sites, including the site of administration, blood, liver, kidney and vascular endothelia, etc. Consequently, peptides and proteins must be protected against degradation in more than one anatomical site for them to reach their target sites intact.

Second, almost all the peptidases and proteases capable of degrading a given peptide or protein are likely to be present in a given anatomical site where the peptide or protein is located (Palmieri & Ward, 1983; Ward, 1984; Palmieri *et al.*, 1985). The implication is that protecting a peptide or protein from degradation by one protease/peptidase may not necessarily lead to marked increase in its stability or in the amount of peptide/protein reaching its site of action (Dodda Kashi & Lee, 1986).

Third, a given peptide or protein is usually susceptible to degradation at more than one linkage within the molecular backbone; each locus of hydrolysis is mediated by a certain peptidase/protease. Often, even when one linkage is modified to circumvent one peptidase/protease, the rest of the peptide molecule is still vulnerable to other peptidases/proteases. This usually manifests itself as a shift in the relative proportion of the various degradation products of a given peptide.

Proteases and peptidases are essentially hydrolyses; hence they have the ability to cleave peptide bonds with the addition of water. Proteases/peptidases rarely show absolute specificity in their action, hence any protease has the potential to hydrolyze more than one substrate (Given *et al.*, 1985). The problem is especially true in the case of kyotorphin. Since kyotorphin is only a dipeptide, it lacks any stereo hindrance which may slow down an unspecific peptidase/protease.

Clearly, in order to promote the delivery of peptides and proteins from any route of administration, the many components of the enzymatic barrier must be controlled. This can be achieved to some extent by modifying the peptide or protein structure, through co-administration of protease inhibitors, or by using the formulation approach.

The Blood-Brain Barrier (BBB) and Its Selective Permeability Properties

The Blood-Brain Barrier (BBB)

The BBB is a membranous barrier that is highly resistant to solute free diffusion and which segregates brain interstitial fluid from the circulating blood. The BBB is comprised of two plasma membranes in series, which are the lumenal and antilumenal membranes of the brain capillary endothelium that are separated by about 0.3 μm of endothelial cytosol.

There are several ultrastructural differences between systemic capillaries and cerebral capillaries which explain the difference in their permeabilities. The main

difference is in the manner how endothelial cells in cerebral capillaries are joined. Cerebral junctions are characterized as tight or closed junctions, which grid the cell circumferentially, forming zona occuludens and acting like a zipper which closes the interendothelial pores that normally exist in microvascular endothelial barrier in peripheral tissue (Rapoport, 1976). This unique architecture prevents the bulk movement of materials between cells and forces compounds to diffuse directly through the phospholipid cell membrane if they are to gain access to the brain parenchyma. A second main difference between cerebral and systemic capillaries is the paucity of vesicles and vesicular transport in the CNS (Brightman, 1977). Vesicular transport is a process for transcellular transport. Vesicles are transported from the luminal to the abluminal membrane. Pinocytotic activity, on the other hand, is concerned with the nutritional requirements of the cell and, as such, involves vesicular movement from the luminal membranes to a cell organelle, presumably by a lysosome. Cerebral endothelial vesicles are usually uncoated and few in number compared to other systems. This lower vesicle content is another mechanism by which the CNS can limit nonspecific influx and have clearly evolved to protect the delicate environment necessary for optimal neural functioning. A third difference is the lack of fenestrae in the cerebral capillaries.

In addition to these structural features, the BBB maintains a number of enzymes which appear to augment its barrier function (Levin, 1977). Since optimal neuronal control requires a careful balancing between neurotransmitter release,

metabolism, and uptake, it is of vital importance to restrict the entry of blood-borne neurotransmitters into the CNS. It is not surprising, therefore, to find high concentrations of such enzymes as catechol-O-methyl-transferase (COMT), monoamine oxidase (MAO), gamma-aminobutyric acid transaminase (GABA-T), and aromatic amino acid decarboxylase (DOPA decarboxylase) in the BBB. The presence of DOPA decarboxylase explains partially the need for giving such large doses of L-dihydroxyphenylalanine (L-DOPA) in the treatment of CNS dopamine deficiencies to achieve appropriate therapeutic cerebral levels. The enzymatic BBB may also play a role in the exclusion of some lipophilic compounds which otherwise might passively diffuse through the barrier.

The Selective Permeability Properties of the BBB

Lipid soluble drugs with a molecular weight of less than 600 readily diffuse through the BBB via lipid mediation based on the high lipid solubility of the drug (Oldendorf, 1974). The prototype example of increasing lipid solubility of a drug compound by masking polar functional groups is the morphine-heroin model. The morphine structure was shown in Figure 1. The relatively low lipophilicity of morphine is attributed to the two hydroxyl groups on the ring nucleus. Methylation of one of these hydroxyl groups results in the conversion of morphine to codeine, and codeine is transported across the BBB approximately tenfold faster than is morphine. Acetylation of both of the morphine hydroxy groups results in the

conversion of morphine to heroin, and heroin is transported through the BBB approximately 100-fold faster than is morphine (Oldendorf *et al.*, 1972).

Some drugs and peptides are bound by plasma proteins. For example, some acidic drugs and peptides are tightly bound to albumin, some lipophilic amine drugs are bound by both albumin and α_1-acid glycoprotein, and some lipid-soluble peptides such as cyclosporin are bound by lipoproteins. These plasma protein-bound drugs may be transported across the BBB by plasma protein-mediated transport. This process arises from enhanced dissociation reactions that occur within the lumen of the capillary and which are catalyzed by transient interactions between the plasma protein and the glycocalyx surface of the brain capillary endothelium (Pardridge, 1987).

Most nutrients in the circulation are water-soluble compounds that would not traverse the BBB in the absence of special carrier-mediated transport systems that are embedded within both the lumenal and antilumenal membranes of the BBB. These carriers and some representative nutrients they transport are listed in Table 4. The substrates listed are not the only substrates those carriers transport.

Insulin can cross the BBB via receptor-mediated peptide transcytosis which involves three sequential steps: 1) receptor-mediated endocytosis at the lumenal or blood side of the BBB, 2) movement of the ligand-receptor complex through the endothelial cytoplasm, and 3) receptor-mediated exocytosis of the ligand into the brain interstitial fluid at the antilumenal or brain side of the BBB.

Table 4. Blood-Brain Barrier Transport Systems and Their Substrates

Carrier	Representative Substrates
Hexose Carrier	Glucose, Mannose, 2-deoxyglucose
Monocarboxylic Acid Carrier	Lactic acid
Amine Carrier	Choline
Neutral Amino Acid Carrier	Neutral amino acids and their analogs e.g. phenylalanine, L-dopa, α-methyl-dopa
Acidic Amino Acid Carrier	Glutamate
Basic Amino Acid Carrier	Arginine, Lysine
Nucleoside Carrier	Adenosine, guanosine, uridine
Purine Base Carrier	Adenine
Thyroid Hormone Carrier	T_3, T_4
Thiamin Carrier	Thiamin

In contrast to carrier-mediated transport of small nutrients, which takes place over a time period of milliseconds to seconds, receptor-mediated transcytosis takes place over a time period of minutes to hours.

In summary, the BBB consists of a relatively impermeable membrane super-imposed on which are mechanisms for allowing the entrance of essential nutrients and the exit of metabolic waste. The permeability of the BBB to molecules in the

general circulation is very selective, which acts to protect the CNS from periodic peripheral changes that might disturb neurofunction.

The Blood-Brain Barrier (BBB) For Peptide and Protein Drug Delivery

The BBB is the main obstacle for the development of centrally active peptides. Since only those agents with sufficient affinity with the lipid membrane will penetrate the BBB, hydrophilic molecules (including peptides) are excluded (Levin, 1980). The BBB is also distinct from the peripheral capillary system in that high concentrations of various lytic enzymes are present, including some highly active neuropeptide degrading enzymes, such as enkephalinase, aminopeptidase, endopeptidase. This enzymatic barrier also prevents the uptake of blood-borne neurotransmitters and neuromodulators (Levin, 1977).

While the BBB acts to protect the CNS from periodic peripheral changes that might disturb neurofunction, it also restricts the movement of many potentially important drugs or hormones, which limits the treatment of cerebral diseases. Varieties of amino acids and peptides have been considered for therapeutic use, such as GABA (aminobutyric acid), DOPA (dihydroxyphenylalnine), enkephalin, KTP, TRH, etc. Unfortunately, the BBB prevents significant uptake of these amino acids/peptides. Therefore, a general method for improving the delivery of amino acids and peptides into the CNS would be desirable.

The overall membrane transport properties are very important for a drug, as these are governing its absorption, distribution, and elimination of its intact form, as well as affecting its binding, affinity, and other important characteristics. One approach for improving brain uptake is via prodrugs.

A prodrug is a pharmacologically inactive compound that results from transient chemical modification of a biologically active specie. When the BBB is considered, increased drug penetration is usually well correlated with the lipophilicity (Levin, 1980). In order to improve the entry of a hydroxy-, amino-, or carboxylic acid-containing drug, esterification or amidation may be performed. This greatly enhances the lipophilicity of the drug. As a result, the drug can better enter the brain parenchyma. Once inside the CNS, hydrolysis of the lipophilic modifying group will release the active compound.

Unfortunately, simple prodrugs suffer from several important limitations. While increasing the lipophilicity of a molecule may increase its chance of crossing the BBB, the uptake of the compound into the other tissue is likewise augmented. This nonselectivity of delivery is detrimental when kyotorphin is considered. Kyotorphin is an enkephalin releasing hormone. Enkephalin receptors are widespread in the human gut, including distal small intestine, colon, cystic duct, gall bladder, pancreas, and esophagus; and are particularly high in pyloric antral mucosa and duodenal G-cells (Uddman *et al.*, 1980). Therefore, the nontarget site toxicity might be exacerbated. In addition, while drug uptake into the CNS is increased, its

efflux is also enhanced. This results in poor tissue retention of the drugs and short biological action. Finally, while the only metabolism associated with prodrugs should be by conversion to the parent drug, other routes can occur that may contribute to the toxicity. These effects, poor selectivity, poor retention, and the possibility of inactive catabolism, often conspire to decrease, not to increase, the therapeutic index of a drug when masked as a prodrug. Therefore, it is very important to find methods which enable delivery of drugs specifically to a particular organ, or site. This requires more than simply optimizing overall membrane transport characteristics. Among the various possible ways to achieve site specific or organ specific delivery, the "chemical delivery system" (CDS) designed by Dr. Bodor is the most flexible and offers possibilities for specific delivery not only to the brain, skin, eye, but to other organs and sites. Properly designed, a CDS should concentrate the desired active agent at its site of action and reduce its concentration in other locations. The main result of this manipulation is not only an increase in the efficacy of the drug entity, but also a decrease in its toxicity.

The Chemical Delivery System (CDS)

As mentioned above, although the acquired lipophilicity in a prodrug approach may be enough to assure the drug of penetrating the BBB into the CNS by passive transport, it does not ensure the drug to stay inside the CNS. Moreover, a simple prodrug approach is prone to the enzymatic barrier. In a perfect approach,

the target drug should acquire enough lipophilicity to penetrate the BBB; and once it is inside the CNS, it should stay inside the CNS. It should also disguise the target peptide/protein enough to confuse the various enzymes during the passage of the substances from the general circulation to the brain.

The CDS is defined as a biologically inert molecule requiring several steps for conversion to the active drug, thereby enhancing drug delivery to the target site (Bodor, 1987).

There are three criteria for a brain-targeting CDS. First, it should be lipophilic enough to allow for brain uptake. Second, after the brain penetration, retention of the lipophilic molecule is required to prevent its efflux from the CNS. Lastly, the conversion intermediate should be degraded enzymatically according to the designed route to release the active molecule over a long period. Since both the BBB and enzymatic degradation prevent the passage of the peptides from the general circulation to the brain tissue, the CDS, an enzyme-based strategy, is an excellent candidate for peptide brain-delivery.

Since it was first proposed by Dr. Bodor in 1978, the brain-targeting CDS has been extensively applied to various neurotransmitters and other pharmaceuticals. Among those, peptides are included as a major consideration.

The Principles of the Redox-Based Chemical Delivery System.

The principle of the CDS that permits enhanced and sustained delivery of drugs to the brain is schematically shown in Figure 2.

The CDS employs interconversion of a lipophilic dihydropyridine moiety to a hydrophilic pyridium salt moiety that is an analog to the NADH $<=>$ NAD^+ co-enzyme system. This lipophilic dihydropyridine carrier is covalently linked to an active drug to form the Drug-CDS that is able to penetrate the BBB. Upon systemic administration, the CDS can partition into several body compartments due to its enhanced lipophilicity; some of those (i.e., the CNS) are inaccessible to the unmanipulated compound. At this point, the CDS is simply working as a lipidal prodrug. The carrier molecule is specially designed, however, to undergo an enzymatically mediated oxidation that converts the membrane-permeable dihydrotrigonellinate (the lipophilic dihydropyridine moiety) to a hydrophilic, membrane-impermeable, trigonellinate salt (the hydrophilic pyridium salt moiety) *in vivo*. The polar carrier-drug conjugate is then trapped behind the BBB. Any of this oxidized salt that is present in the periphery will be rapidly lost as it is now an excellent candidate for elimination by kidney and liver. The conjugate that is trapped behind the BBB then slowly hydrolyzes to release the active drug in a sustained manner.

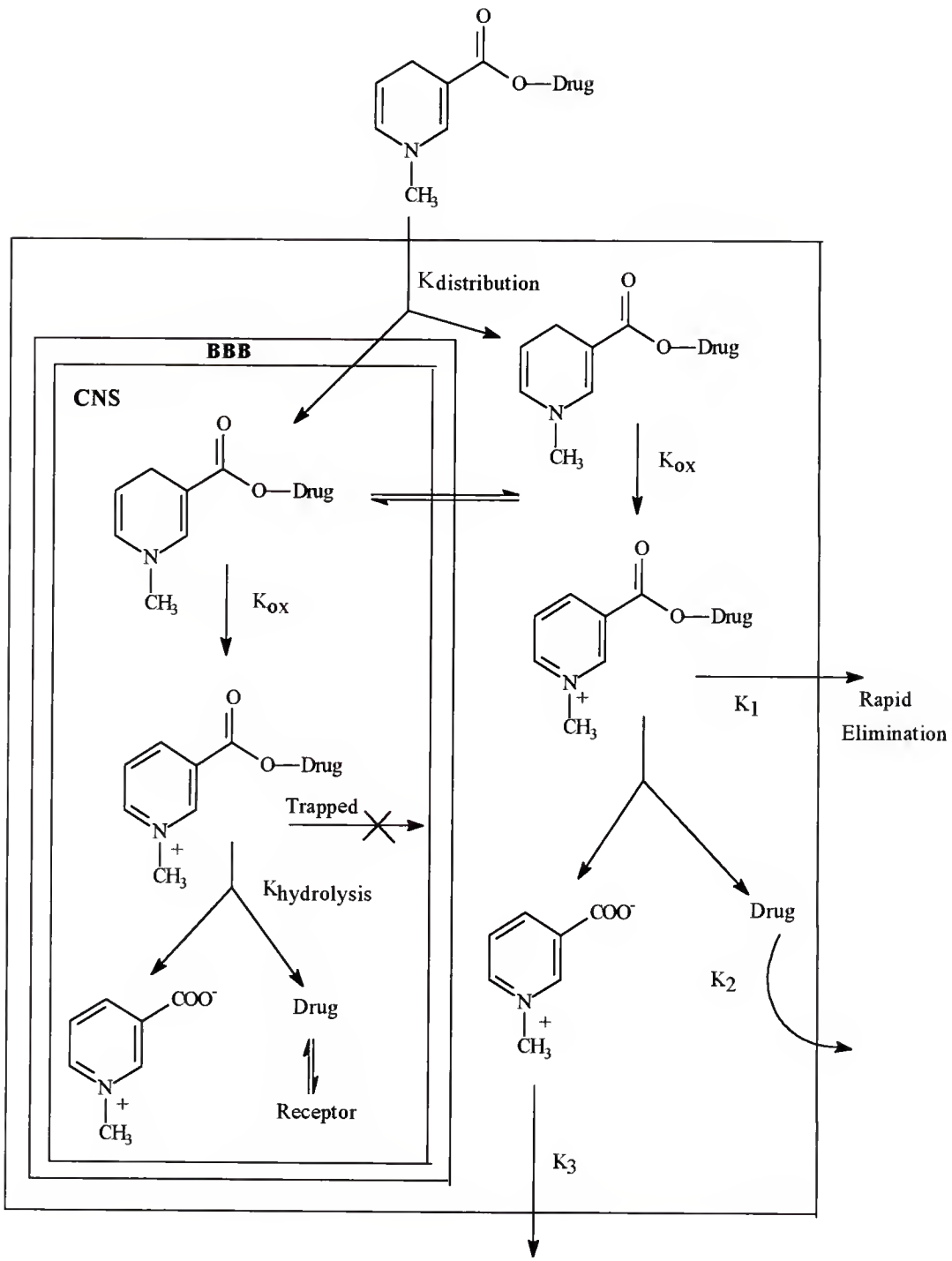


Figure 2. The Mechanism of the Redox-Based CDS for Enhanced and Sustained Delivery of Drugs to the Brain

By the system design, concentration of the active drug is low in the periphery, which minimizes dose-related side effects and toxicity. In addition, the active drug in the CNS is present mostly as an inactive conjugate, which offers two advantages -- lower central toxicity and increased dose interval.

Delivery of Peptides into the CNS via the CDS

For many smaller drugs, a simple redox targetor has proved to be applicable (Bodor & Brewster, 1983; Bodor & Simpkins, 1983). For peptides, however, the attachment of 1,4-dihydrotrigonelline to the NH_2-terminus alone will not furnish sufficient increase in lipophilicity and will only protect peptides against aminopeptidases. The unmodified COOH-terminal of the molecule will decrease the lipophilicity as well as be susceptible to cleavage by numerous exo- and endo-peptidases. In the strategy called "molecular packaging," the peptide unit of the CDS appears only as a perturbation on the bulky molecule dominated by lipophilic modifying groups that assure the BBB penetration and irrecognition by peptidases.

A chemical delivery system for peptide brain-delivery has been proposed based on all the considerations, as shown in Figure 3. A centrally active peptide sequence (P) is placed in a molecular package that disguises its peptide nature and provides biolabile, lipophilic functions to penetrate the BBB by passive transport. The design incorporates an 1,4-dihydrotrigonellinate targetor (T) at the NH_2-terminal of the peptide via a spacer (S) and a cholesteryl ester at the C-terminal.

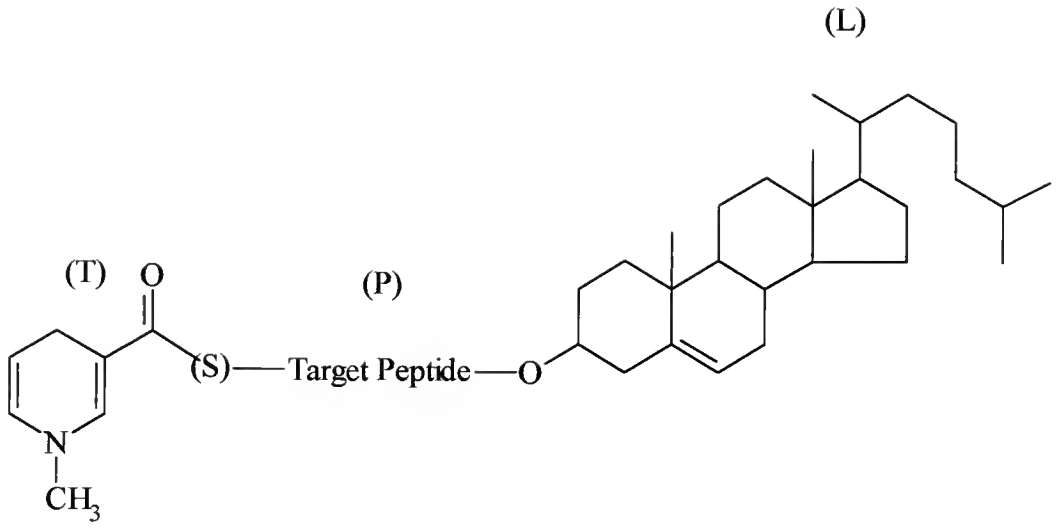


Figure 3. The Chemical Delivery System for Peptides

Because of the low amidase activity of the brain tissue, a spacer (S) is used to separate the peptide sequence (P) from the targetor part of the CDS. The spacer is selected based on the peptidolytic activity prevalent at the site of action, so that the release of the desired peptide is favored over the degradation induced by other peptidases. The brief description of its metabolism was shown in Figure 4.

After the molecular package penetrates the BBB and enters the CNS, the 1,4 dihydrotrigonellinate targetor (T) undergoes an enzymatically-mediated oxidation to become a hydrophilic, membrane impermeable trigonellinate salt (T^+) which traps the whole molecule (II) behind the BBB and inside the CNS. Hydrolysis of this molecule provides a polar targetor-peptide conjugate (III) which is a substrate for peptide degrading enzymes, such as dipeptidyl peptidases and post proline cleaving enzymes.

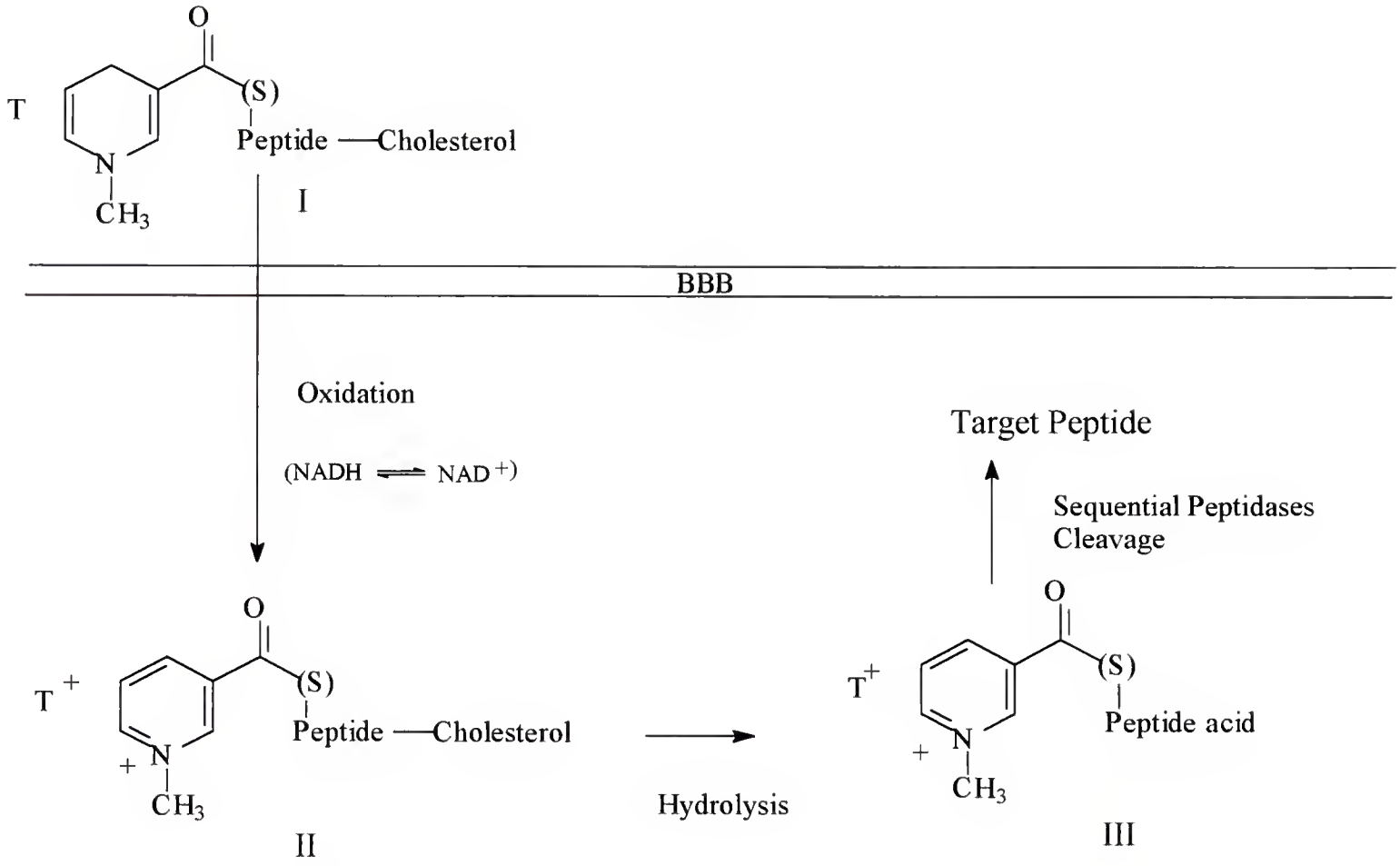


Figure 4. The Sequential Metabolism of the CDS of Peptide

Through the sequential enzymatic degrading, the final biologically active peptide is released in a pharmacologically significant amount inside the brain. The spacer is used to ensure a precise cleavage between the peptide and the targetor, not among the amino acid residues of the parent peptide.

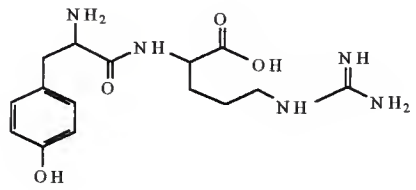
The Chemical Delivery Systems for Kyotorphin and a Kyotorphin Analog

Basic amino acids, such as arginine and lysine, have a basic side-chain amine group which is positively charged at pH 7.4 -- the pH *in vivo*. The side-chain group make basic amino acids very hydrophilic and therefore it is more difficult for them to penetrate the BBB than other amino acids. Since kyotorphin also has a very short half-life in plasma (Ueda *et al.*, 1985; Akasaki & Tsuji, 1991), it is very challenging to try to deliver kyotorphin or its analog into the brain.

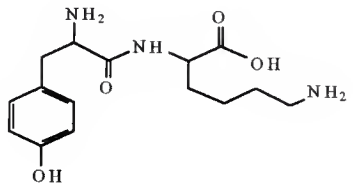
A possible approach is the derivatization of the peptide to produce a transport form that is markedly more lipophilic than the parent peptide as well as resistant towards the various peptidases. Yet, it must remain cleavable by enzyme-catalyzed hydrolysis at a prescribed joint to have the sustained brain-specific release of the parent peptide *in situ*.

The efficacy of this kind of peptide delivery system depends not only on the effective lipophilicity of the molecular package, but also on minimal exposure of the target peptide to vascular peptidases. Therefore, the CDS approach seems to be an excellent candidate for brain-targeted delivery of kyotorphin.

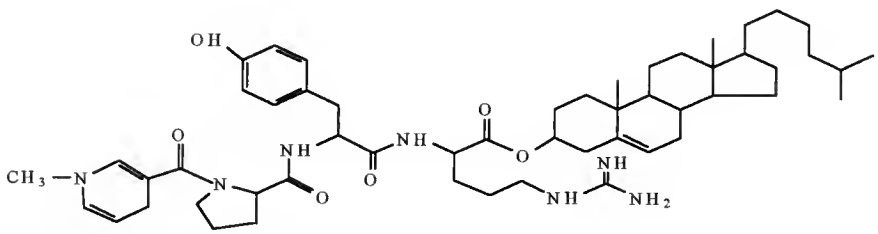
The structures of KTP (kyotorphin) and KAYK (Kyotorphin Analog -- Tyrosyl-Lysine) and their basic CDS's are shown in Figure 5.



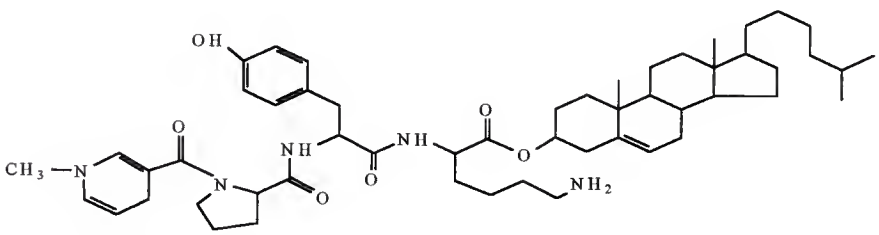
Kyotorphin (L-Tyr-L-Arg)



L-Tyr-L-Lys (a Kyotorphin analog)



1,4-Dihydrotrigonellyl-Pro-Tyr-Arg-Cholesteryl Ester



1,4-Dihydrotrigonellyl-Pro-Tyr-Lys-Cholesteryl Ester

Figure 5. The Structure of KTP, KAYK, and Their Basic CDS's

The proline is used as the spacer that may be replaced by alanine. Double amino acids may also be used as the spacer.

Since the side-chain NH_2 groups of arginine and lysine are very basic (pKa > 10), they would exist mostly positively charged *in vivo* (pH ~ 7.4), which would decrease the lipophilicity of the whole molecular package. Therefore, additional protection should be considered to ensure the desired high-lipophilic nature of the CDS. Such a CDS for KAYK (Tyr-Lys) with a proline as the spacer is shown in Figure 6.

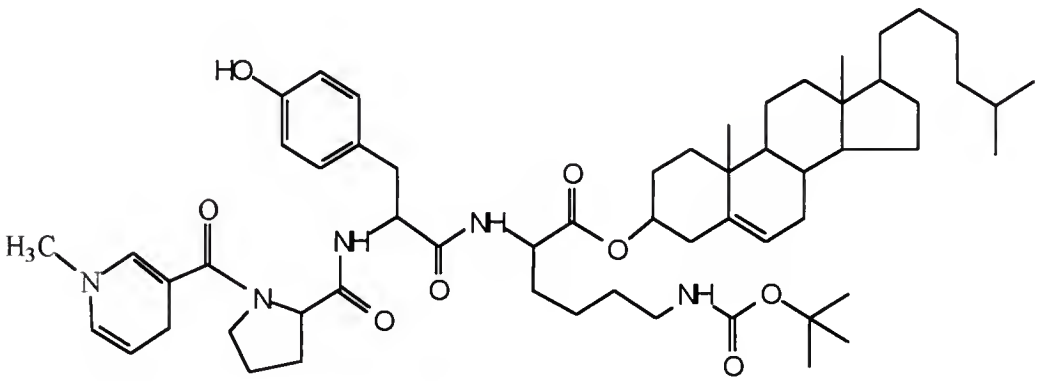


Figure 6. The Structure of CDS-P--a CDS for KAYK
1,4-Dihydrotrigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester

The side chain NH_2 group of lysine offers another way to deliver KAYK into the CNS which is unsuitable for Tyr-Arg, as shown in Figure 7.

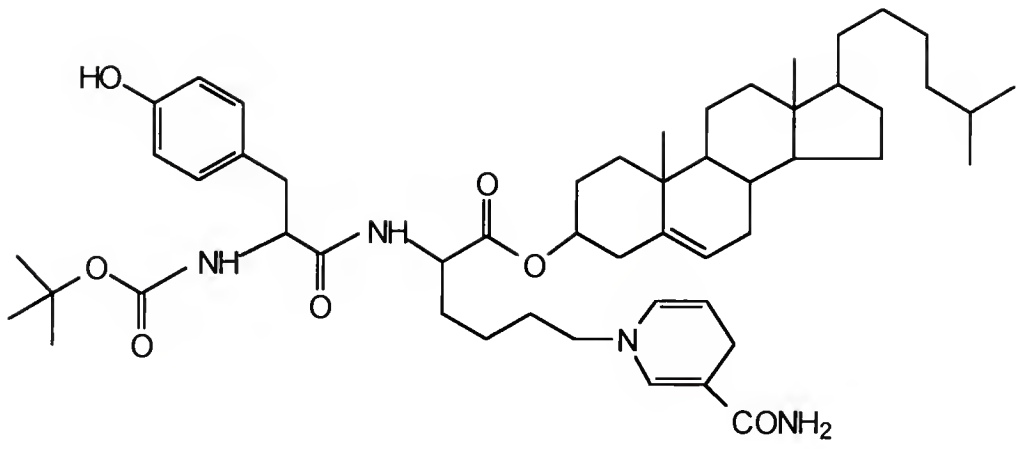


Figure 7. The Structure of Boc-Tyr-Nys-Cholesteryl Ester (BTRA)

In this molecular package, the lysine moiety of KAYK is replaced by Nys -- the redox analog of the natural diamino acid lysine. The replacement is applicable theoretically due to the isoelectronic/isosteric effects of Tyr-Lys and Tyr-Nys *in vivo*, as shown in Figure 8. The whole molecular package is called KAYK brain-targeted redox analog (BTRA).

Compared to arginine, lysine is less basic and is easier to convert to potential bioreversible lipophilic derivatives, such as CDS and BTRA.

When KTP-CDS/KAYK-CDS/BTRA is administered systemically, as discussed in the scheme for the general CDS (Fig. 2), KTP/KAYK-CDS/BTRA can partition into several body compartments; some of those are inaccessible to the unaltered KTP/KAYK. The design of CDS/BTRA allows for an enzymatically

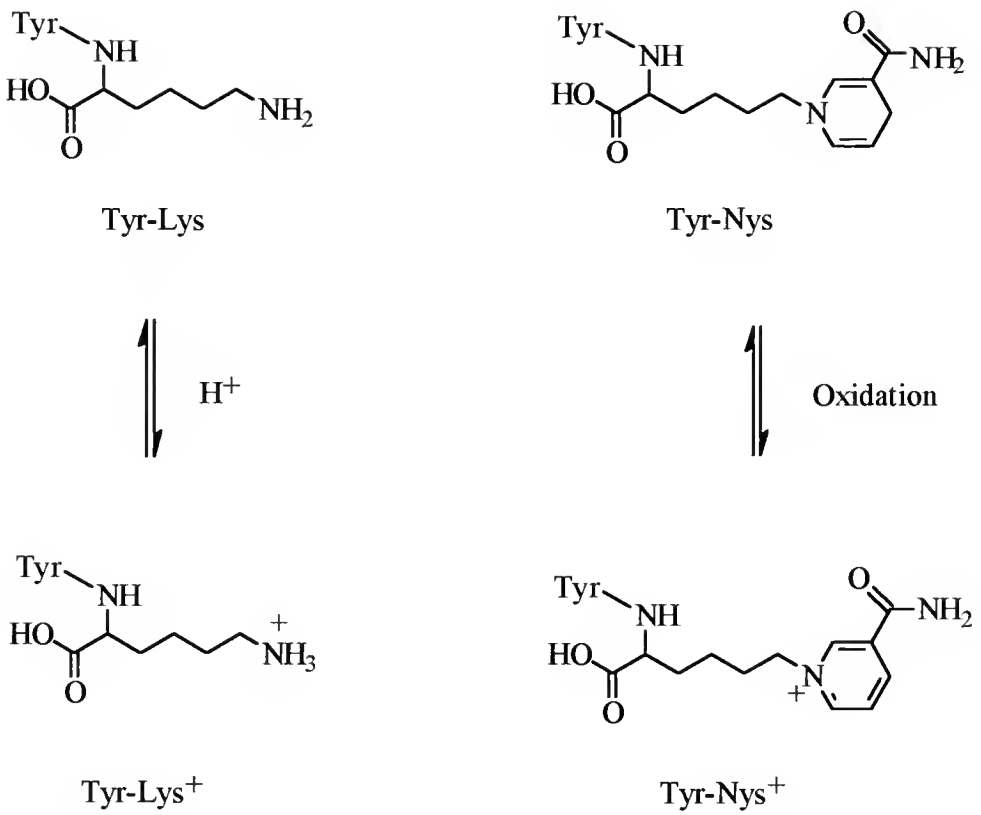


Figure 8. The Isoelectronic/isosteric Effects of Tyr-Lys and Tyr-Nys *in vivo*

mediated oxidation that converts the membrane-permeable dihydrotrigonellinate to a membrane-impermeable hydrophilic trigonellinate salt, a reaction that occurs throughout the organism. The polar salt is then trapped behind the BBB and is held within the CNS. The peripherally distributed polar salt is rapidly eliminated via kidney and liver. For example, in the case of CDS-P, the polar CNS trapped drug -- Trigonellyl-L-Pro-L-Tyr-L-Lys(Boc)-Cholesteryl Ester will be slowly hydrolyzed to

release Tyrosyl-lysine inside the CNS over a sustained time. Since the peripheral concentration of Trigonellyl-L-Pro-L-Tyr-L-Lys(Boc)-Cholesteryl Ester is very low, the systemic toxicity is minimized. The sequential metabolism of CDS-P is shown in Figure 9, and those of CDS-PA, CDS-PP, and BTRA are shown in Figure 10-12.

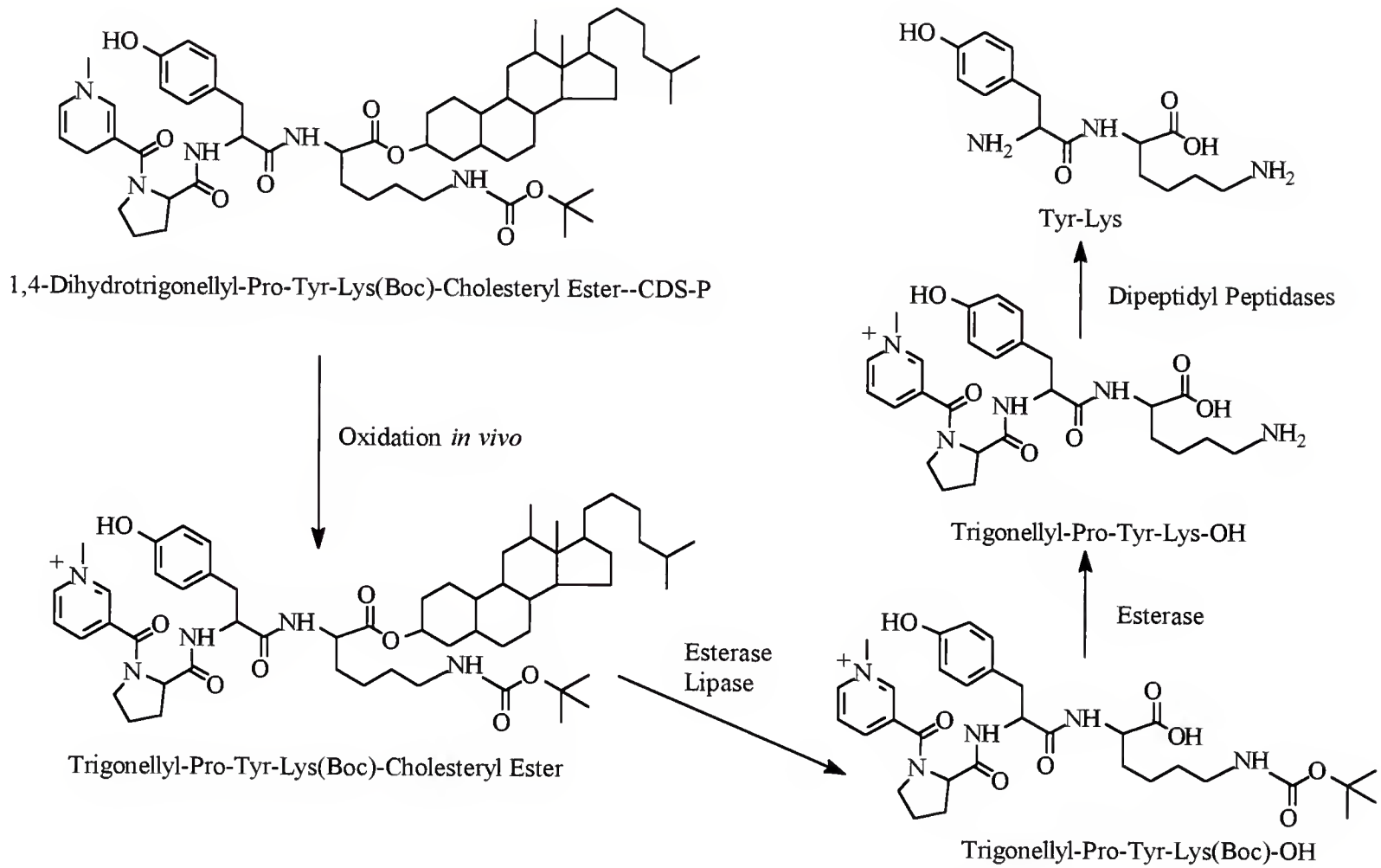


Figure 9. The Sequential Metabolism of CDS-P *in vivo*.

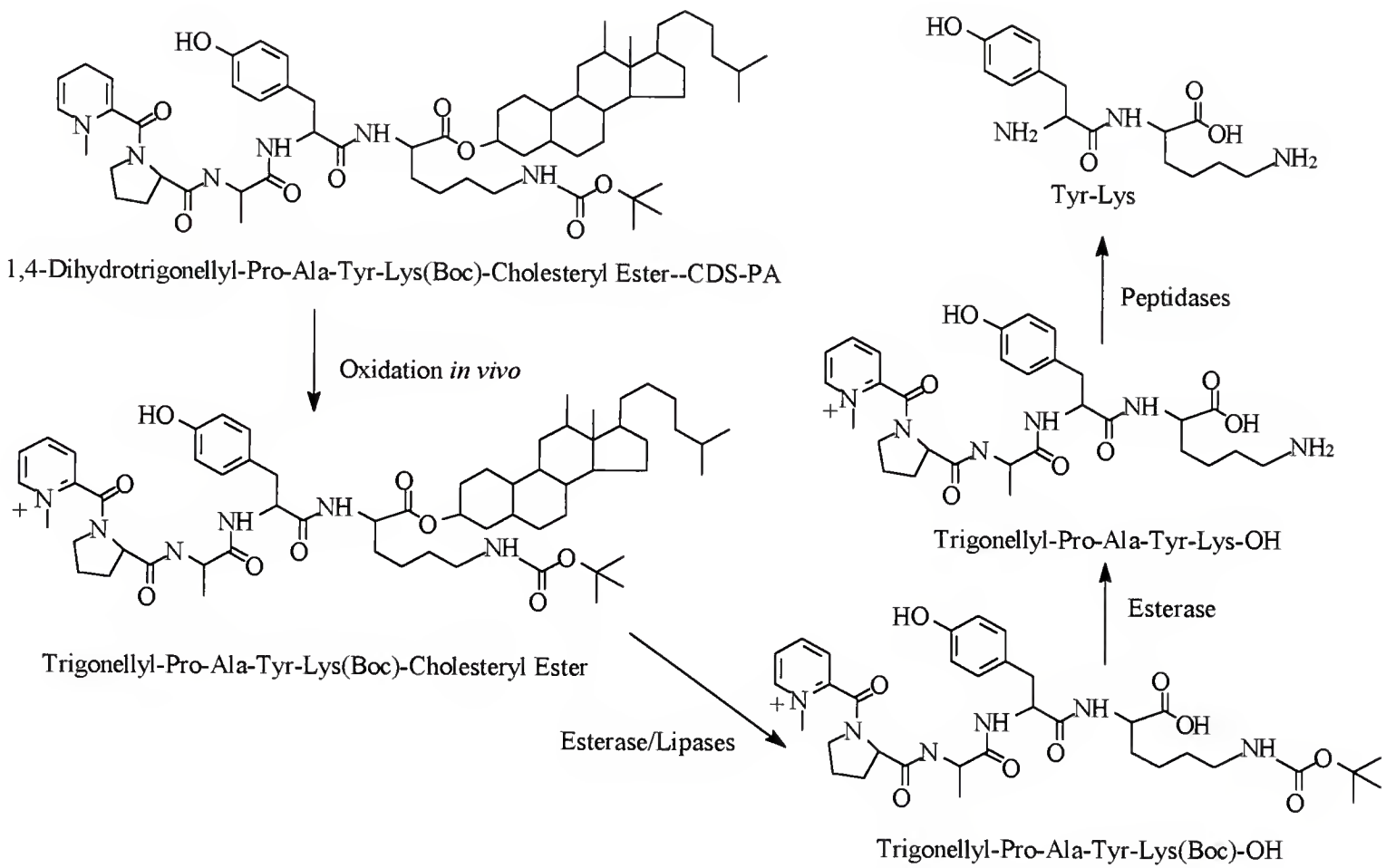


Figure 10. The Sequential Metabolism of CDS-PA *in vivo*.

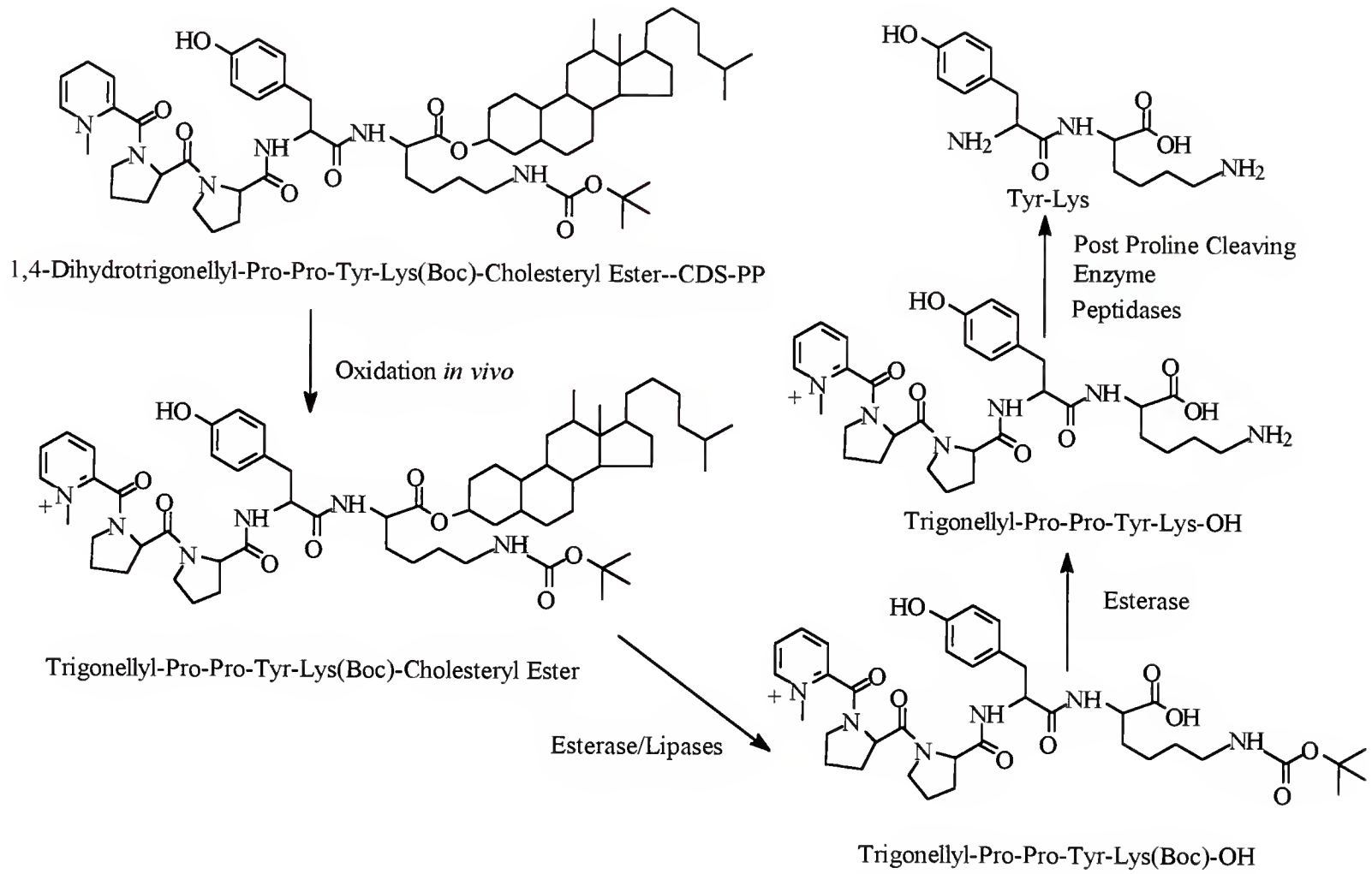


Figure 11. The Sequential Metabolism of CDS-PP *in vivo*.

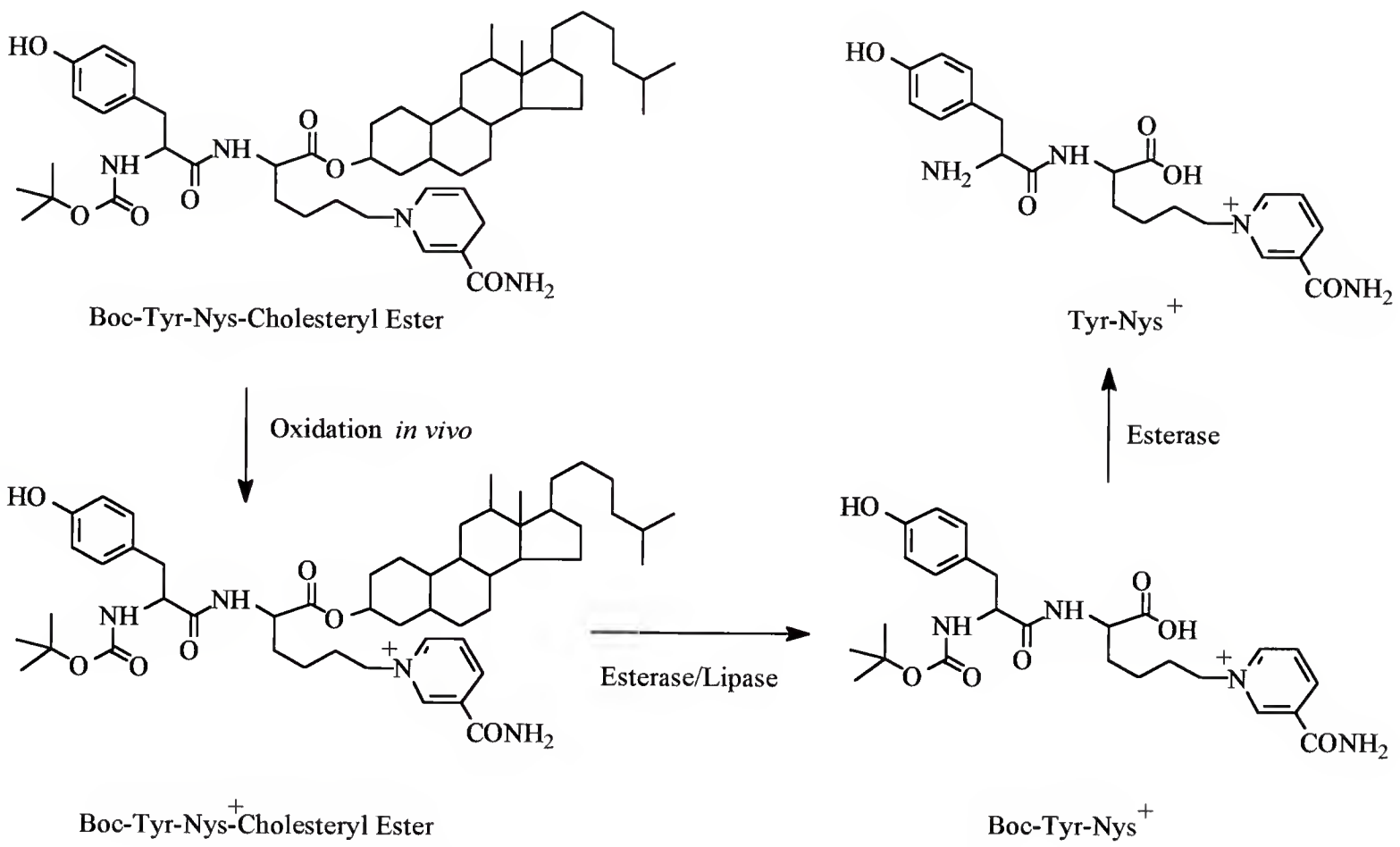


Figure 12. The Sequential Metabolism of BTRA *in vivo*.

CHAPTER 2
MATERIALS AND METHODS

Materials

All chemicals used were reagent grade or peptide synthesis grade. All solvents used were A.C.S. Reagent Grade. Amino acid derivatives were L-configured and purchased from BaChem Inc., Torrance, CA. All solvents were purchased from Fisher Scientific.

Melting points were taken on a Fisher-Jones melting point apparatus and are uncorrected. All synthesized products were characterized by FAB (fast atom bombardment) mass spectroscopy by using a Kratos MS80RFA Mass Spectrometer, Manchester, U. K. TLC (thin layer chromatography) determinations were carried out on silica-gel 60 coated foil -- Merck DC-Alufolien Kiesegel (silica gel) 60 F_{254}. Elemental analyses of compounds synthesized were performed by Atlantic Microlab, Inc., Atlanta, GA.

Regular column chromatography was performed using silica gel (100-200 mesh) and appropriate mobile phase; flash column chromatography was performed using silica gel (200-400 mesh) and appropriate mobile phase under the pressure of 10 psi.

Synthetic Protocol for the CDS's (Chemical Delivery Systems) of Kyotorphin

Both Boc and Fmoc methods were used to synthesize the CDS's for the brain-targeted delivery of the kyotorphin. There were six CDS's projected: 1) CDS with one alanine as the spacer (CDS-KTP-A, Fig. 13), 2) CDS with double alanine (Ala-Ala) as the spacer (CDS-KTP-AA); 3) CDS with Pro-Ala as the spacer (CDS-KTP-PA); 4) CDS with one proline as the spacer (CDS-KTP-P); 5) CDS with double prolines (Pro-Pro) as the spacer (CDS-KTP-PP); and 6) CDS with Ala-Pro as the spacer (CDS-KTP-AP).

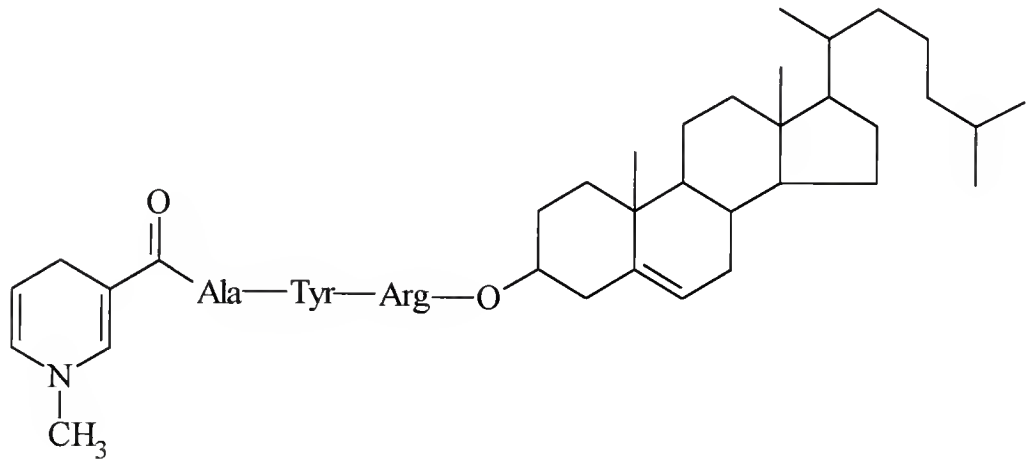


Figure 13. CDS-KTP-A

The synthetic scheme of KTP-CDS-A by using the Boc method is shown in Figure 14.

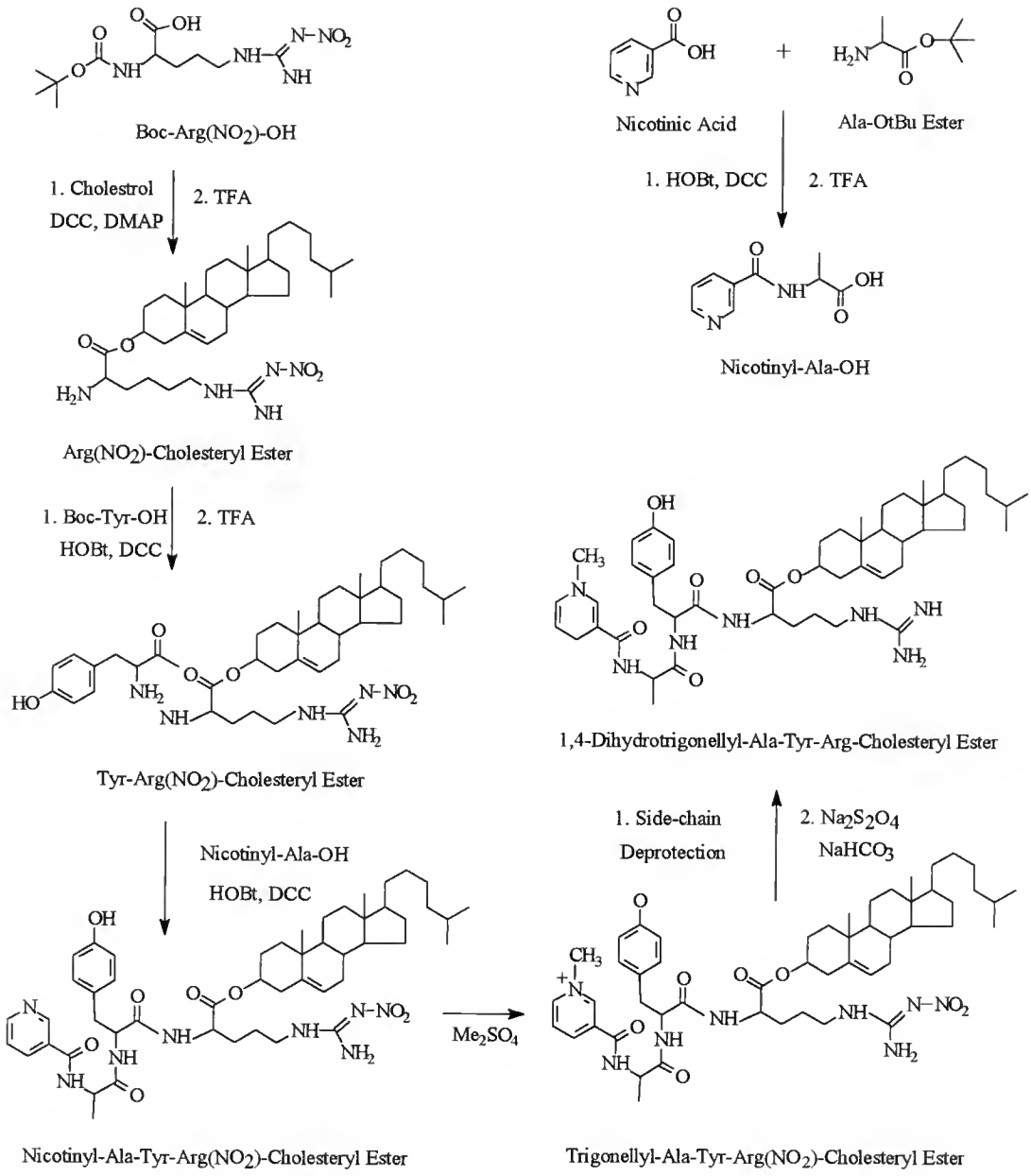


Figure 14. The Synthetic Scheme for KTP-CDS-A
by Using the Boc Method

Ala-OtBu ester was replaced by Pro-OtBu ester for the synthesis of Nicotinyl-Pro-OH in the synthesis of 1,4-Dihydrotrigonellyl-Pro-Tyr-Arg-Cholesteryl Ester (KTP-CDS-P). An extra amino-acid residue (alanine or proline) was added at the N-terminal of NH_2-Tyr-Arg(NO_2)-Cholesteryl Ester and then coupled with Nicotinyl-Ala-OH or Nicotinyl-Pro-OH during the synthesis to generate the other four KTP-CDS's.

The synthetic scheme of the KTP-CDS-A by using the Fmoc method is shown in Figure 15. Ala-OtBu ester was replaced by Pro-OtBu ester for the synthesis of Nicotinyl-Pro-OH in the synthesis of 1,4-Dihydrotrigonellyl-Pro-Tyr-Arg-Cholesteryl Ester (KTP-CDS-P). An extra amino-acid residue (alanine or proline) was added after the finish of NH_2-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester during the synthesis to generate the other four KTP-CDS's.

Synthetic Protocol for CDS's (Chemical Delivery Systems) and BTRA (Brain Targeted Redox Analog) of Tyr-Lys

The Fmoc method was used to synthesize the brain-targeted delivery systems of Tyr-Lys. There are four brain-targeted delivery systems to be synthesized: 1) CDS with one proline as the spacer (CDS-P), 2) CDS with double prolines as the spacer (CDS-PP), 3) CDS with Pro-Ala as the spacer (CDS-PA), and 4) BTRA. Their structures are shown in Figure 16. The synthetic scheme of CDS-P was shown in Figure 17.

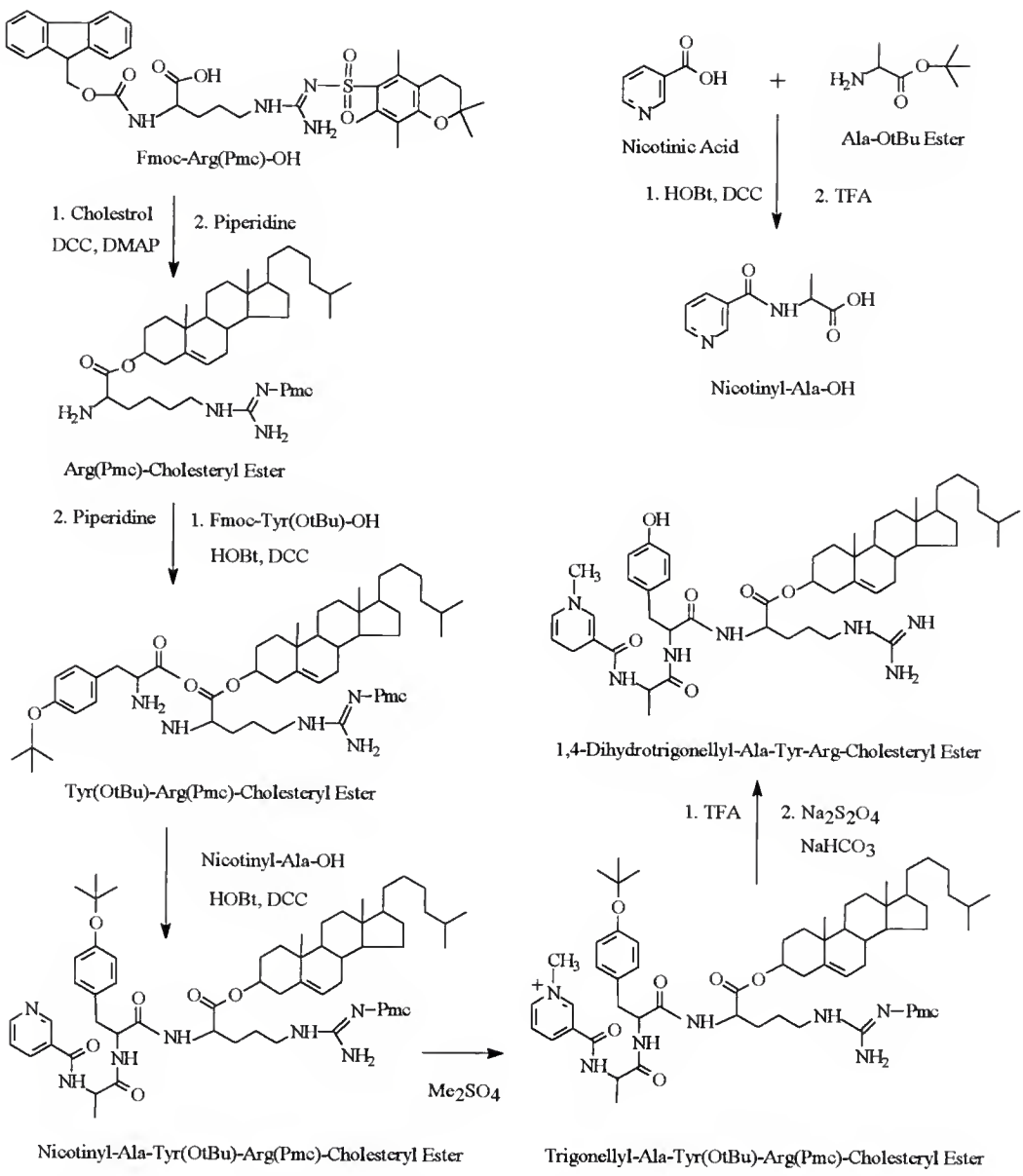
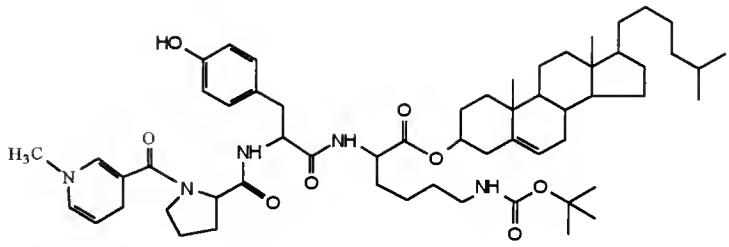
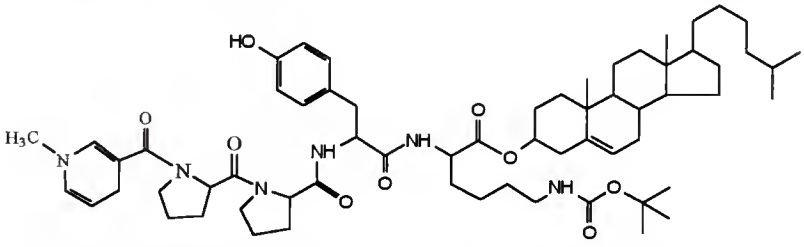


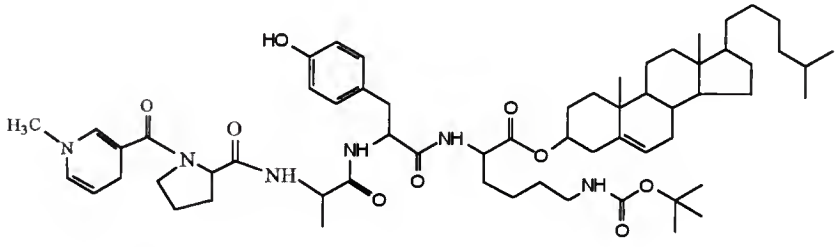
Figure 15. The Synthetic Scheme for KTP-CDS-A
by Using the Fmoc Method



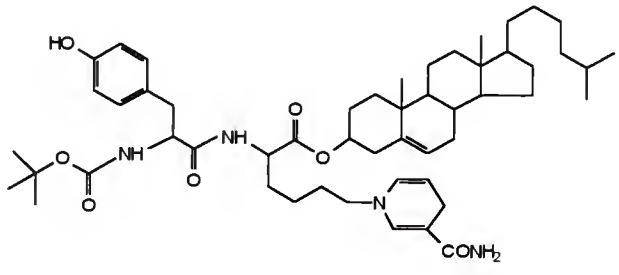
1,4-Dihydrotrigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester -- CDS-P



1,4-Dihydrotrigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester -- CDS-PP



1,4-Dihydrotrigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester -- CDS-PA



Boc-Tyr-Nys-Cholesteryl Ester -- BTRA

Figure 16. The Structures of KAYK-CDS's & BTRA

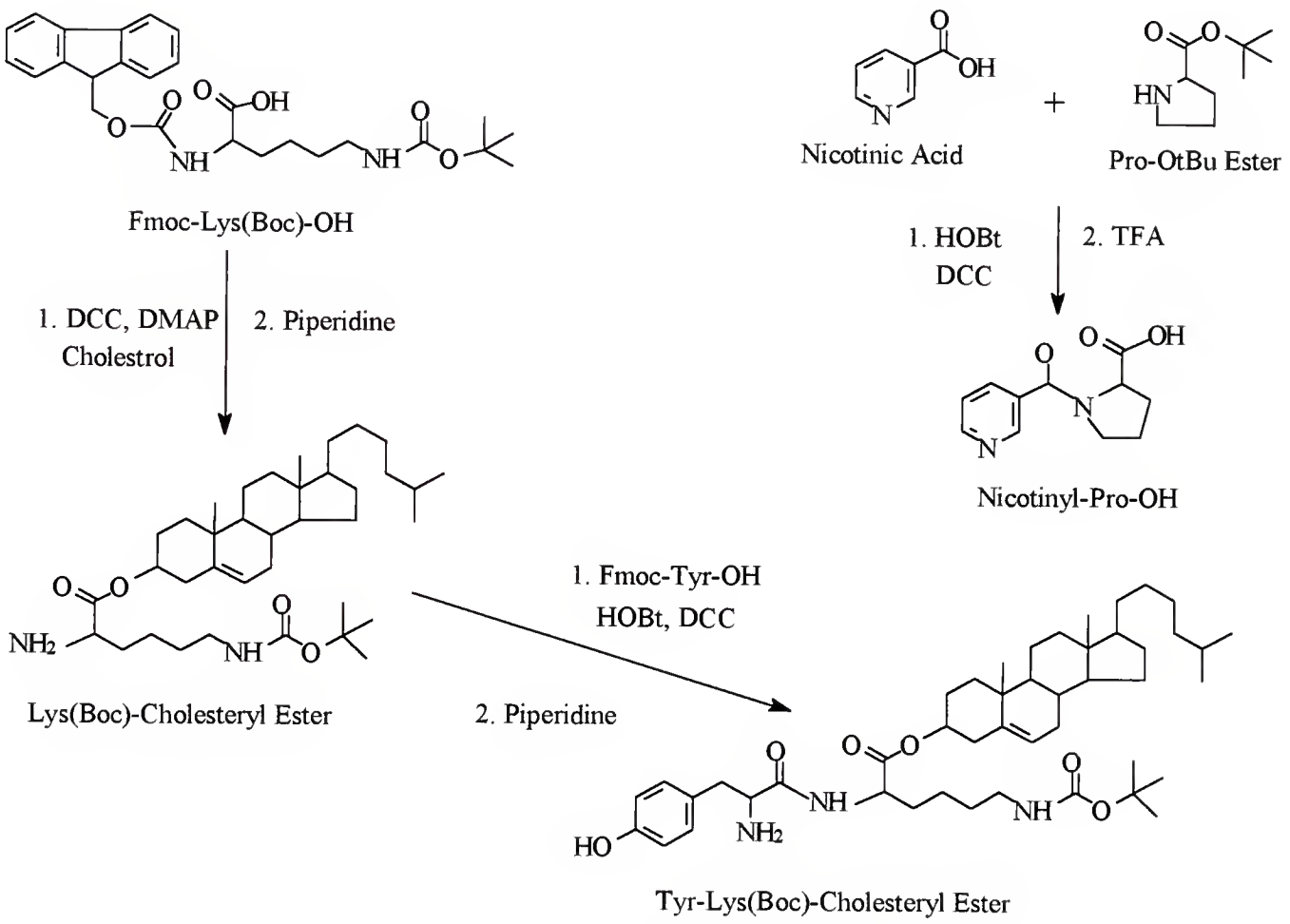


Figure 17. The Synthetic Scheme for CDS-P

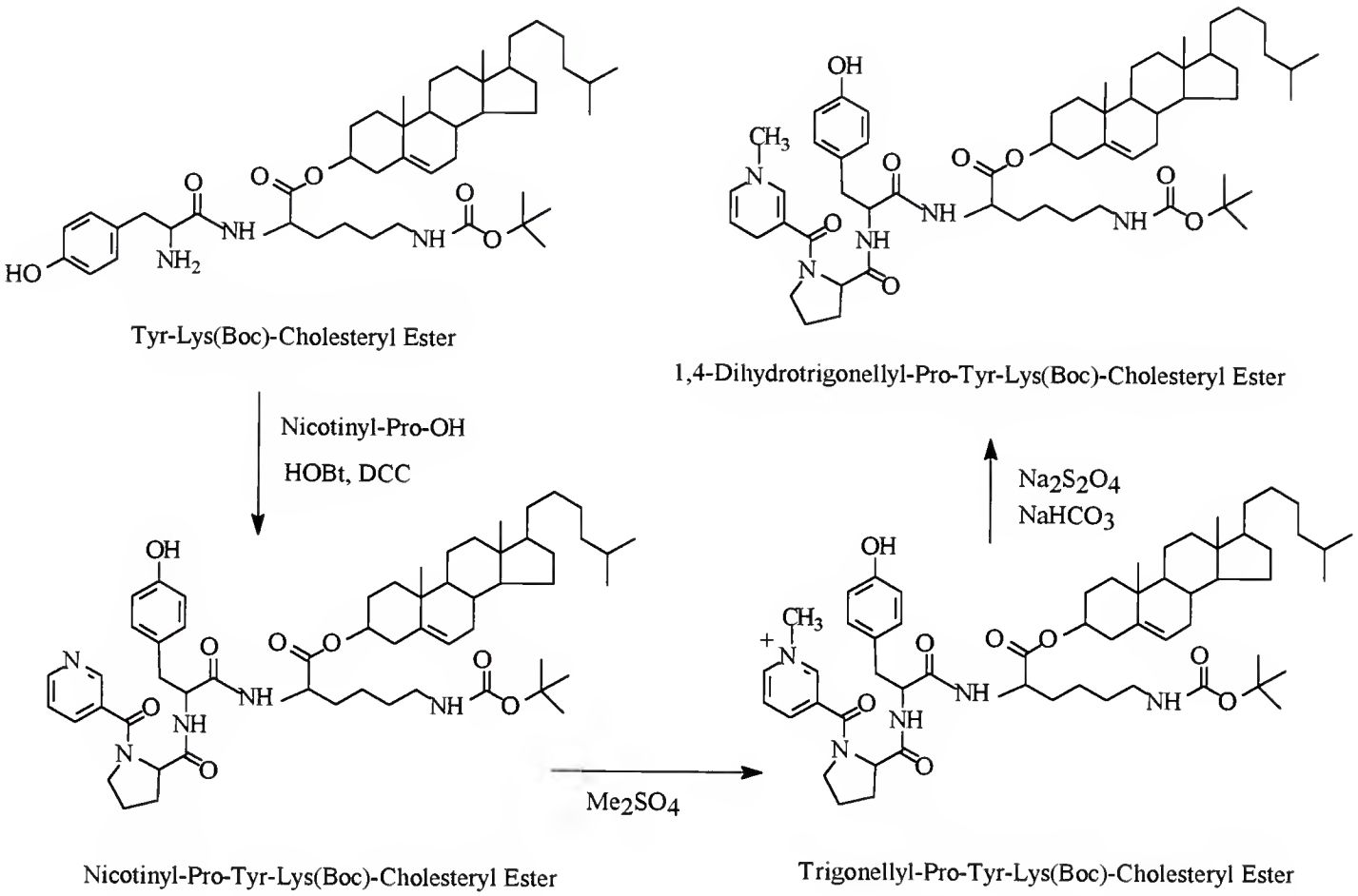


Figure 17 ------ continued

An extra amino-acid residue (alanine or proline) was added at the N-terminal of Tyr- Lys(Boc)-Cholesteryl Ester and then coupled with Nicotinyl-Pro-OH during the synthesis to generate CDS-PA and CDS-PP -- the other two CDS's of the Lys-Tyr. The synthetic scheme of BTRA is shown in Figure 18.

Typical Experimental Procedures for Chemical Synthesis

To prepare cholesteryl esters of Arg(NO_2/Pmc) and Lys(Boc/Fmoc), DCC (dicyclohexylcarbodiimide) was used as the dehydrating agent and DMAP (dimethylaminopyridine) was used as the catalyst. Throughout the synthesis, all amide bonds were coupled by using the DCC/HOBt method. The Fmoc and Boc groups were removed by Piperidine/CH_2Cl_2 (1:3) and TFA/CH_2Cl_2 (1:1), respectively. The Pmc and O-tBu groups were removed by TFA/H_2O (19:1). The N-alkylation in preparation of the trigonellyl compounds was performed by using dimethyl sulfate (Me_2SO_4) in ethyl ether or methalene chloride. Nys^+ was formed by Zincke reaction (Fig. 19). The reduction of the quaternary salts to the corresponding 1,4-dihydrotrigonellinate derivative was carried out by using sodium dithionite ($Na_2S_2O_4$) as the reducing agent in a mixture of methanol and deaerated aqueous sodium bicarbonate ($NaHCO_3$).

In all cases, the intermediates synthesized were purified by silica gel chromatography, except for those otherwise noted. In order to avoid side-reactions, all the coupling reactions were performed at an initial temperature of 0°C and then

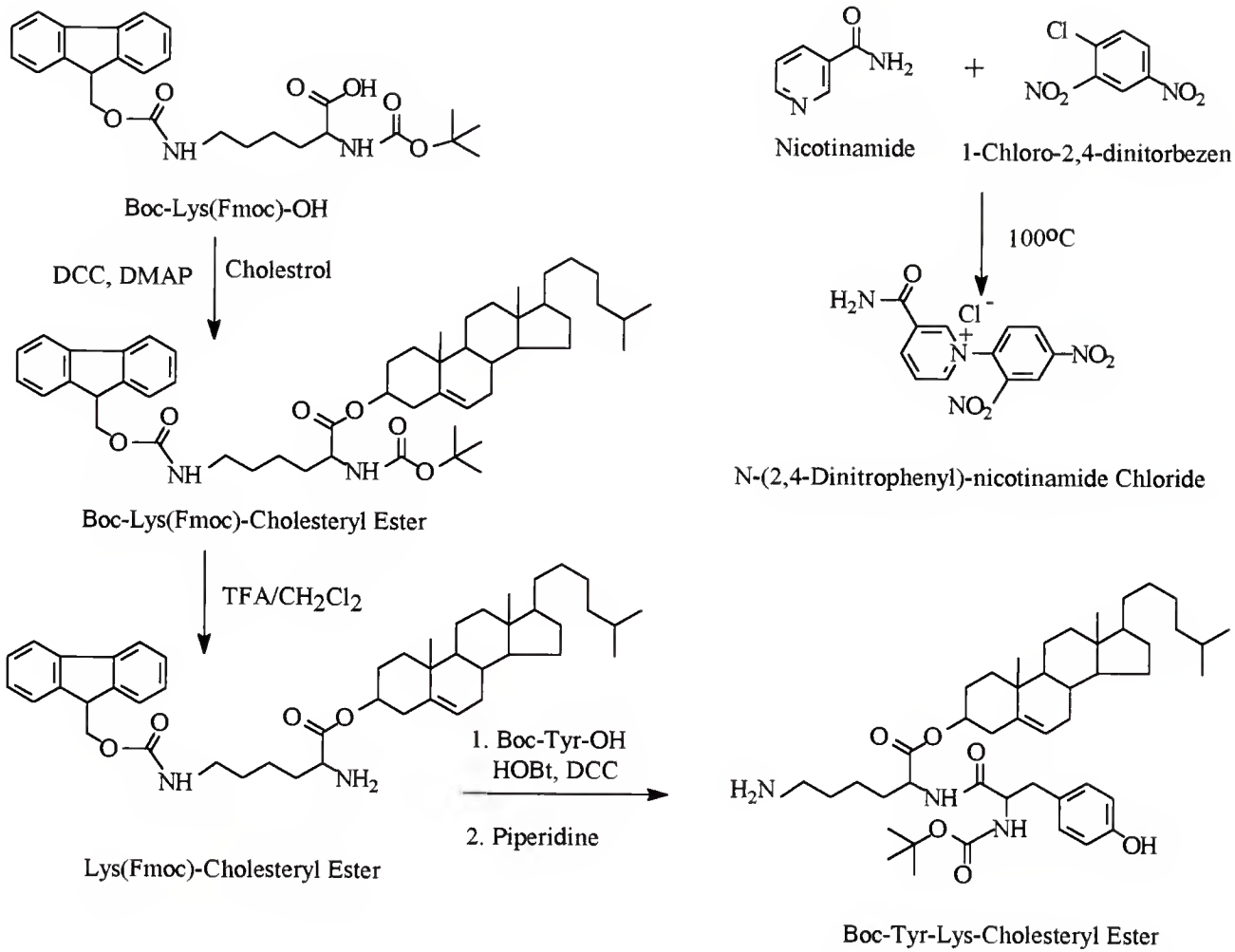


Figure 18. The Synthetic Scheme for BTRA

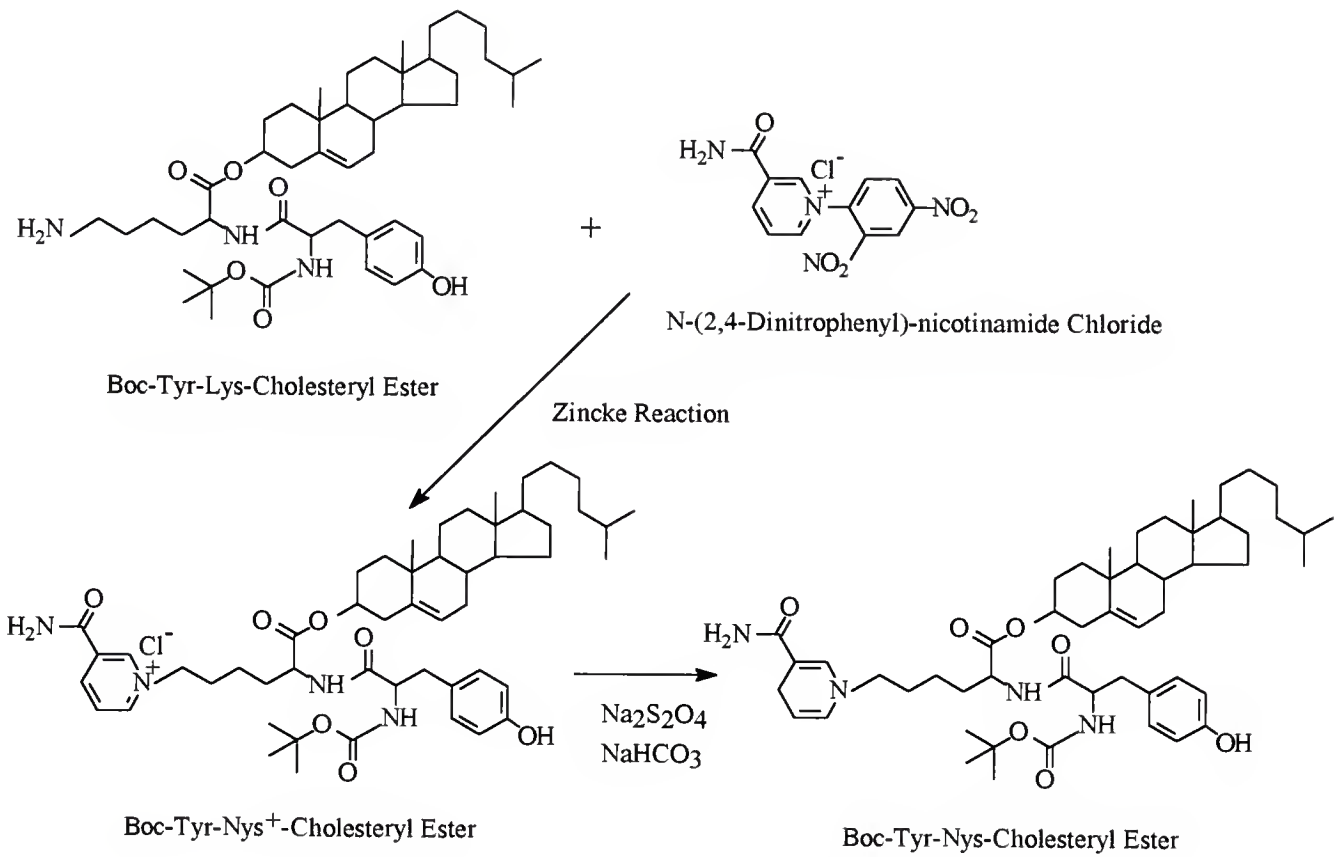


Figure 18 ------ continued

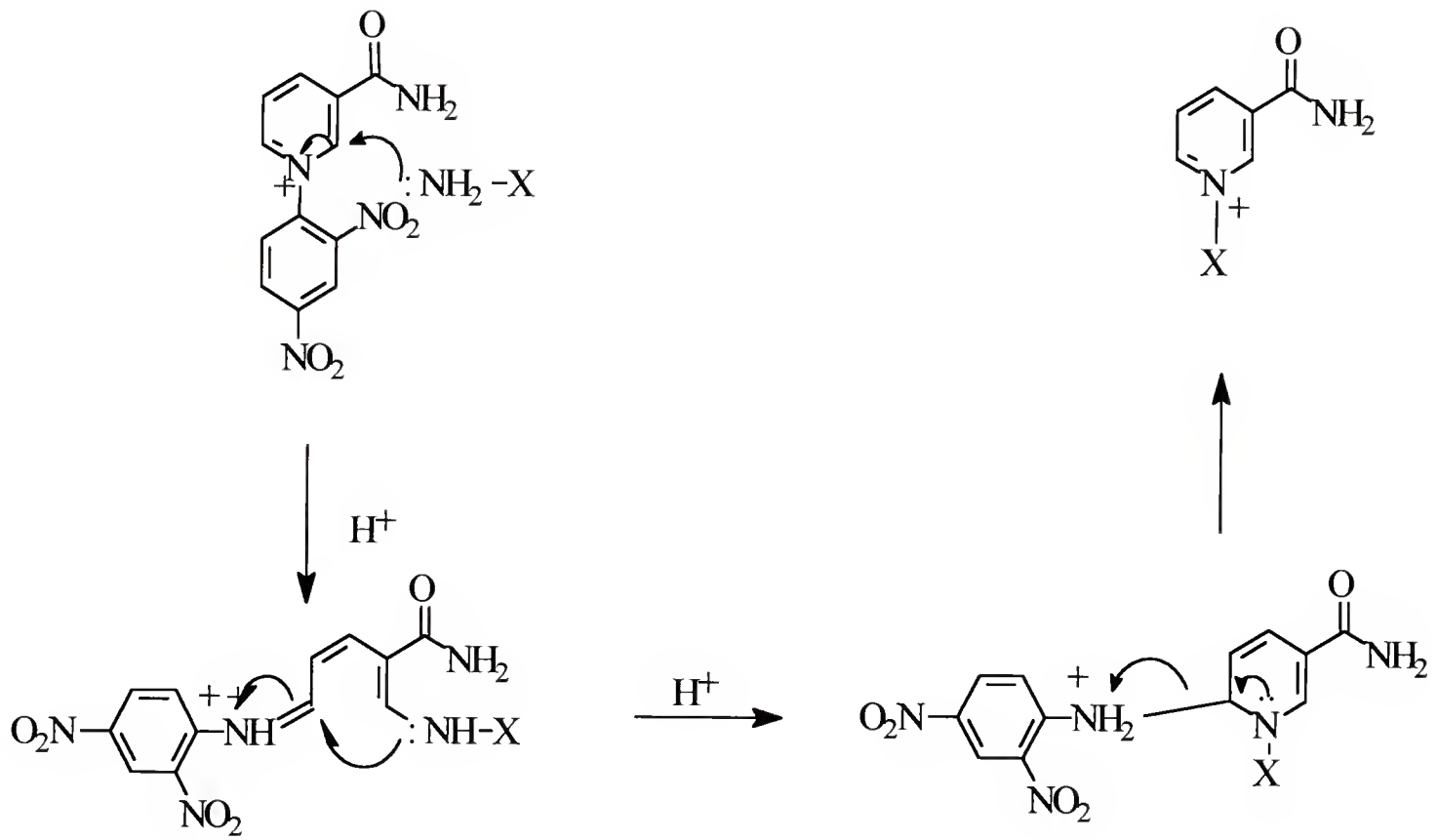


Figure 19. The Mechanism of Zincke Reaction

at room temperature. The N-methylation was performed at room temperature with an excess of the alkylating agent. The reduction of the quaternary salts with sodium dithionite was accomplished at 0°C in an oxygen-free environment.

A liquid-phase peptide synthesis method has been applied for the syntheses of all KAYK-CDS's and BTRA. The liquid-phase method has been selected because it is most suitable for the synthesis of peptides of less than 10 amino-acid residues or of peptides with the carboxyl terminal which is not amenable to solid-phase techniques (such as a labile cholesteryl ester). The other advantages of the liquid-phase technique are that it is very easy to scale-up to multi-gram scale and much less expensive compared to solid-phase technique.

Pharmacology Studies

The Animals

Male Sprague-Dawley rats weighing 250-300 g were purchased from Harlan Sprague Dawley Inc. (Indianapolis, Indiana, USA) and used in all the experiments. Animals were housed one per cage in room temperature (~ 25°C) on a 14 hour light cycle. Purina lab Chow and water were provided *ad libitum*.

All the animal studies were conducted in accordance with the guidelines set forth in the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH-80-23).

The Testing Method

Drugs were dissolved in vehicle (PG/DMSO, 2:1) and were injected into the animals through the tail vein. When administered icv., the minimum dose of kyotorphin which can produce the analgesic effect in rat is about 1 mg/kg (Rolka *et al.*, 1983). Accordingly, CDS-P at doses of 0.0030, 0.0075, 0.0148, 0.0223 mmol/kg (equimolar to 1.0, 2.5, 5.0, and 7.5 mg/kg of kyotorphin) were administered to monitor the dose-response. Vehicle and 0.0223 mmol/kg of drugs (equimolar to 7.5 mg/kg of kyotorphin) were also administered to study the pharmacological activities of the other KAYK brain-targeted delivery systems and their important intermediates.

Tail-flick latency, an index of spinal cord mediated analgesia (D' Amour & Smith, 1941), was measured to evaluate the analgesic effects of the brain-targeted delivery systems for KAYK. Time between presentation of a focused beam of light and the reflexive removal of the tail from the stimulus was defined as the tail-flick latency period (T), and the tail-flick latency difference between each time point and control was defined as the change in tail flick latency. In the absence of response, a cut-off period of 1 min was used. Each drug at each dose was tested on six animals.

Control latency (T_0) was obtained 10 minutes prior to the drug administration; the test latencies (T_{15}, T_{30}, T_{60}, T_{120}, T_{180}, T_{240}, T_{300}, T_{360}) were measured at 15 min, 30 min, 1 hr, 2, hr, 3 hr, 4 hr, 5 hr, and 6 hr after the administration of drugs. The instrument used was a Model 33 Tail Flick Analgesia

Meter (Litc, Inc., Landing, N. J.). The beam dial was set at 90 and the sensitivity was at eight.

The Effect of Naloxone on CDS-PP and BTRA Induced Analgesia

To test the effect of naloxone on the KAYK brain-targeted delivery systems, rats were treated with the CDS-PP and BTRA (0.0223 mmol/kg) as described previously; and the tail-flick latencies were recorded at 15 and 30 minutes after the administration of the drugs. Subsequently, naloxone hydrochloride, 2 mg/kg, dissolved in 0.9% saline, was injected subcutaneously, and the tail-flick latency periods were recorded at 45, 60, 90, and 120 minutes (15, 30, 60, and 90 minutes after the administration of naloxone).

Statistical Analysis

Anova was used to process all the data generated from the pharmacological experiments.

Generally, analysis of variance, or anova, is a statistical procedure used to determine whether means from two or more samples are drawn from populations with the same mean. Anova is one of the most powerful and useful statistical procedure utilized in the analysis of research data. The analysis of variance allows us to make what is known as *a test of significance* between or among means, the results being given on the basis of the probability. The test of significance is cast in

the form of accepting or rejecting what is termed a *null hypothesis*, the hypothesis of no difference between or among means. If the null hypothesis were rejected, we are guided by the fact that the probability of finding a difference as large as or larger than that obtained in the experiment is quite small ($p \leq 0.05$ or $p \leq 0.01$), and we concluded that there is a significant difference between the treatment means. If the null hypothesis is rejected at the 5 percent level ($p \leq 0.05$), we state that there is a "significant" difference between two means. If the null hypothesis is rejected at the 1 percent level ($p \leq 0.01$), we state that there is a "highly significant" difference between two means (Damon & Harvey, 1987).

Single-Factor Anova performs simple analysis of variance, which tests the hypothesis that means from several samples are equal. Two-Factor with Replication Anova performs an extension of the single-factor anova that includes more than one sample for each group of data. In all the anova performed in this study, the α value was set at 0.05.

Single-Factor Anova was used to compare the maximum analgesic effects (the mean of maximum response) induced by KAYK brain-targeted delivery systems with those of vehicle, kyotorphin, intermediates of CDS-PP and BTRA and among KAYK brain-targeted delivery systems themselves.

Two-Factor with Replication Anova was used to compare the analgesic effects (the mean of maximum response) induced by KAYK brain-targeted delivery

systems across the testing period with those of vehicle, kyotorphin, intermediates of CDS-PP and BTRA and among KAYK brain-targeted delivery systems themselves.

The variances of the data were measured by standard deviation.

CHAPTER 3
CHEMICAL SYNTHESIS

Synthesis of Kyotorphin-CDS's (the Boc- Method)

Synthesis of Arg(NO_2)-Cholesteryl Ester

N-*a*-Boc-Arginine(NO_2)-OH (3.72 g, 10.0 mmol) in 75 ml CH_2Cl_2/DMF (9:1) was stirred at 0°C and cholesterol (3.88 g, 10.0 mmol) dissolved in 75 ml CH_2Cl_2 was added, which was followed immediately by DCC (2.44 g, 11.0 mmol) dissolved in 15 ml CH_2Cl_2 and DMAP (1.68 g, 14.0 mmol) dissolved in 25 ml CH_2Cl_2. The mixture was stirred for 48 hours at room temperature. The DCU formed was filtered off and the solvent was removed *in vacuo*. The crude material was dissolved in $CHCl_3$ and washed successively with 10% citric acid solution (3x100 ml), 5% $NaHCO_3$ solution (3x100 ml), and saturated NaCl solution (100 ml). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to give a solid -- Boc-Arg(NO_2)-Cholesteryl Ester. The product was dissolved in 60 ml CH_2Cl_2/TFA (1:1) and stirred at room temperature for half an hour. Then the solvent was removed *in vacuo* to minimum and precipitated from ethyl ether to give a white solid -- Arg(NO_2)-Cholesteryl Ester (3.62 g, 51.81%). TLC R_f=0.35, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=611 $(M+Na)^+$.

Synthesis of Tyr-Arg(NO_2)-Cholesteryl Ester

Arg(NO_2)-Cholesteryl ester (3.50 g, 5.0 mmol) in 35 ml CH_2Cl_2/DMF (9:1) was stirred with Et_3N (0.53 g, 5.0 mmol) at 0°C. Boc-Tyr-OH (1.7 g, 5.0 mmol) dissolved in 35 ml CH_2Cl_2/DMF (1:1) was added, which was followed immediately by DCC (1.22 g, 5.5 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.91 g, 6.75 mmol) dissolved in 15 ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU formed was filtered off and the solvent was removed *in vacuo*. The crude material was dissolved in $CHCl_3$ and washed successively with 10% citric acid solution (3x100 ml), 5% $NaHCO_3$ solution (3x100 ml), and saturated NaCl solution (100 ml). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to give a solid -- N-*a*-Boc-Tyr-Lys(NO_2)-Cholesteryl Ester. The peptide ester was deprotected with TFA/CH_2Cl_2 (1:1) in an identical manner illustrated in the preceding step to give a white solid -- Tyr-Arg(NO_2)-Cholesterol Ester (3.53 g, 81.1%). TLC R_f=0.56, CH_3OH/CH_2Cl_2 (1:8); Mass spectrum: m/z=774, $(M+Na)^+$.

Synthesis of Nicotinyl-Ala-OH

Ala-OtBu ester•HCl (1.82 g, 10.0 mmol) and Et_3N (1.01 g, 10.0 mmol) in 25 ml CH_2Cl_2 were stirred at 0°C. Nicotinic acid (1.85 g, 15.0 mmol) dissolved in 25 ml DMF was added, which was followed immediately by DCC (2.42 g, 11.0 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (1.82 g, 13.0 mmol) dissolved in 15

ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU formed was filtered off and the solvent was removed *in vacuo*. The crude material was dissolved in CH_2Cl_2 and washed successively with 10% citric acid solution (3x100 ml), 5% $NaHCO_3$ solution (3x100 ml), and saturated NaCl solution (100 ml). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to give a solid -- Nicotinyl-Ala-OtBu. The product was dissolved in 50 ml 95% TFA in H_2O and stirred for one hour at room temperature. Then the majority of the solvent was removed and the material was recrystallized from ethyl ether to give a white solid -- Nicotinyl-Ala-OH (1.56 g, 80.41%). TLC R_f=0.16, CH_3OH/CH_2Cl_2 (1:9); Mass spectrum: m/z=195 $(M+H)^+$.

Synthesis of Nicotinyl-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester

Tyr-Arg(NO_2)-Cholesteryl Ester (1.5 g, 2.0 mmol) and Et_3N (0.2 g 2.0 mmol) dissolved in 25 ml CH_2Cl_2/DMF (9:1) were stirred at 0°C. Nicotinyl-Ala-OH (0.39 g, 2.0 mmol) in 25 ml DMF/CH_2Cl_2 (1:1) was added, which was followed immediately by DCC (0.44 g, 2.2 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.38 g, 2.7 mmol) dissolved in 10 ml DMF. The mixture was stirred for 96 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo*. The material obtained was dissolved in CH_2Cl_2 and washed successively with 10% citric acid solution (3x100 ml), 5% $NaHCO_3$ solution (3x100 ml), and saturated NaCl solution (100 ml). The organic phase was dried over

anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Nicotinyl-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester (1.32 g, 67.28%). TLC R_f=0.37, CH_3OH/CH_2Cl_2 (1:9); Mass spectrum: m/z=928 $(M+H)^+$.

Synthesis of Trigonellyl-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester

Nicotinyl-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester (0.49g, 5.0 mmol) in 50 ml CH_2Cl_2 was stirred at 0°C, dimethylsulfate (0.32 g, 25 mmol) was added. The mixture was stirred for overnight at room temperature. The solvent was evaporated to minimum and the product was precipitated from ethyl ether to afford a white solid -- Trigonellyl-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester (0.45 g, 91.84%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=942, M^+.

Synthesis of Ala-Tyr-Arg(NO_2)-Cholesteryl Ester

Tyr-Arg(NO_2)-Cholesteryl ester (4.33 g, 5.0 mmol) and Et_3N (0.53 g, 5.0 mmol) in 35 ml CH_2Cl_2/DMF (9:1) were stirred at 0°C. Boc-Ala-OH (0.67 g, 5.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (1.22 g, 5.5 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.91 g, 6.75 mmol) dissolved in 10 ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo*. The crude material was dissolved in $CHCl_3$ and washed successively with

10% citric acid solution (3x100 ml), 5% $NaHCO_3$ solution (3x100 ml), and saturated NaCl solution (100 ml). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to give a solid -- N-*a*-Boc-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester. The peptide ester was deprotected with TFA in CH_2Cl_2 (1:1) in an identical manner illustrated in the previous step to give a white solid -- Ala-Tyr-Arg(NO_2)-Cholesterol Ester (3.69 g, 78.2%). TLC R_f=0.41, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=845, $(M+Na)^+$.

Synthesis of Nicotinyl-Ala-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester

Ala-Tyr-Arg(NO_2)-Cholesteryl Ester (1.65 g, 2.0 mmol) and Et_3N (0.2 g 2.0 mmol) dissolved in 25 ml CH_2Cl_2/DMF (9:1) were stirred at 0°C. Nicotinyl-Ala-OH (0.39 g, 2.0 mmol) in 25 ml DMF/CH_2Cl_2 (1:1) was added, which was followed immediately by DCC (0.44 g, 2.2 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.38 g, 2.7 mmol) dissolved in 10 ml DMF. The mixture was stirred for 96 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo*. The material obtained was dissolved in $CHCl_3$ and washed successively with 10% citric acid solution (3x100 ml), 5% $NaHCO_3$ solution (3x100 ml), and saturated NaCl solution (100 ml). The organic phase was dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid --

Nicotinyl-Ala-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester (1.32 g, 67.28%). TLC R_f=0.45, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=999 $(M+H)^+$.

Synthesis of Kyotorphin-CDS's (the Fmoc Method)

Synthesis of Arg(Pmc)-Cholesteryl Ester

N-*a*-Fmoc-Arg(Pmc)-OH (6.60 g, 10.0 mmol) in 100 ml CH_2Cl_2 was stirred at 0°C and cholesterol (3.88 g, 10.0 mmol) dissolved in 75 ml CH_2Cl_2 was added, which was followed immediately by DCC (2.44 g, 11.0 mmol) dissolved in 15 ml CH_2Cl_2 and DMAP (1.68 g, 14.0 mmol) dissolved in 25 ml CH_2Cl_2 . The mixture was stirred for 48 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo* to give a solid -- N-*a*-Fmoc-Arg(Pmc)-Cholesteryl Ester. The product was dissolved in 60 ml Piperidine/CH_2Cl_2 (1:3) and was stirred at room temperature for half an hour. Then the solvent was removed *in vacuo*. The crude material was dissolved in $CHCl_3$ and washed with distilled water (100 ml) three times, dried over anhydrous Na_2SO_4, and evaporated to give a solid. Silica-gel column chromatography (7% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Arg(Pmc)-Cholesteryl Ester (5.68 g, 70.4%). TLC R_f=0.50, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=809 $(M+H)^+$.

Synthesis of Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester

Arg(Pmc)-Cholesteryl Ester (4.04 g, 5.0 mmol) in 35 ml CH_2Cl_2 was stirred at 0°C and Fmoc-Tyr(OtBu)-OH (2.32 g, 5.0 mmol) dissolved in 35 ml CH_2Cl_2 was added, which was followed immediately by DCC (1.22 g, 5.5 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.91 g, 6.75 mmol) dissolved in 15 ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo* to give a solid -- N-*a*-Fmoc-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester. The peptide ester was deprotected in 35 ml Piperidine/CH_2Cl_2 (1:3) in an identical manner as illustrated in the previous step. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester(3.88 g, 75.4%). TLC R_f=0.45, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1029 $(M+H)^+$.

Synthesis of Nicotinyl-Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester

Nicotinyl-Ala-OH (0.39 g, 2.0 mmol) in 25 ml DMF/CH_2Cl_2 (1:1) was stirred at 0°C and Tyr(O-tBu)-Arg(Pmc)-Cholesteryl-Ester (2.05 g, 2.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (0.44 g, 2.2 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.38 g, 2.7 mmol) dissolved in 10 ml DMF. The mixture was stirred for 96 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo*. The material obtained was dissolved in CH_2Cl_2 and washed with 1N HCl (3x100 ml),

5% $NaHCO_3$ (3x100 ml), and saturated NaCl solution (100 ml), respectively. Then the organic phase was dried over anhydrous Na_2SO_4 and removed *in vacuo*. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Nicotinyl-Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester (1.43 g, 60.91%). TLC R_f=0.57, CH_3OH/CH_2Cl_2 (1:9); Mass spectrum: m/z=1205 $(M+H)^+$.

Synthesis of Trigonellyl-Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester

Nicotinyl-Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester (0.50 g, 0.41 mmol) in 25 ml CH_2Cl_2 was stirred at 0°C and dimethylsulfate (0.26 g, 20 mmol) was added. The mixture was stirred for overnight at room temperature. The solvent then was removed at reduced pressure. The product was dissolved in CH_3OH and recrystallized from ethyl ether to afford a white solid -- Trigonellyl-Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester (0.475 g, 95.12%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:9; Mass spectrum: m/z=1219, M^+.

Synthesis of Trigonellyl-Ala-Tyr-Arg-Cholesteryl Ester

Trigonellyl-Pro-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester (0.25 g, 0.21 mmol) was dissolved in 25 ml TFA/H_2O (19:1) and stirred at 0°C for an hour. Then the solvent was removed at reduced pressure and the product was triturated with Et_2O three times and was recrystallized from Et_2O to afford a white solid -- Trigonellyl-

Ala-Tyr-L-Arg-Cholesteryl Ester (0.15 g, 79.72%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:9); Mass spectrum: m/z=896, M^+.

Synthesis of 1,4-Dihydrotrigonellyl-Ala-Tyr-Arg-Cholesteryl Ester

Trigonellyl-Ala-Tyr-Arg-Cholesteryl Ester (0.1 g, 0.11 mmol) in 15 ml H_2O was stirred at 0°C under argon and $NaHCO_3$ (0.092 g, 1.1 mmol) and Na_2SO_4 (0.184 g, 1.1 mmol) was added little by little. The mixture was stirred at 0°C under argon for an hour. Then saturated NaCl solution (50 ml) was added to dilute the solution which was then extracted with 25 ml CH_2Cl_2. The organic phase was separated, washed with deaerated water several times, dried over anhydrous Na_2SO_4, and evaporated to afford a yellow solid -- 1,4-Dihydrotrigonellyl-Ala-Tyr-Arg-Cholesteryl Ester (0.056 g, 56.2%). TLC R_f=0.33, CH_3OH/CH_2Cl_2 (1:9). UV spectrum showed the typical absorption peak for dihydro-compounds at 350 nm.

Synthesis of Nicotinyl-Pro-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester

Nicotinyl-Ala-OH (0.22 g, 1.0 mmol) in 15 ml DMF/CH_2Cl_2 (1:1) was stirred at 0°C and Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester (0.99 g, 1.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (0.22 g, 1.1 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.19 g, 1.35 mmol) dissolved in 10 ml DMF. The mixture was stirred for 96 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo*. The

material obtained was dissolved in CH_2Cl_2 and washed with 1N HCl (3x100 ml), 5% $NaHCO_3$(3x100 ml), and saturated NaCl (100 ml) solution, respectively. Then the organic phase was dried over anhydrous Na_2SO_4 and removed *in vacuo*. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Nicotinyl-Pro-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester (0.71 g, 57.61%). TLC R_f=0.51, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1231, $(M+H)^+$.

Synthesis of Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester

Tyr(OtBu)-Arg(Pmc)-Cholesteryl ester (2.5 g, 2.5 mmol) in 35 ml CH_2Cl_2 was stirred at 0°C and Fmoc-Ala-OH (0.78 g, 2.5 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (0.61 g, 2.75 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.46 g, 3.38 mmol) dissolved in 15 ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo* to give a solid -- N-*a*-Fmoc-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester. The peptide ester was deprotected in 35 ml Piperidine/CH_2Cl_2 (1:3) in an identical manner illustrated in the previous step. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester (2.54 g, 77.75%). TLC R_f=0.47, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1101, $(M+H)^+$.

Synthesis of Nicotinyl-Ala-Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester

Nicotinyl-Ala-OH (0.39 g, 2.0 mmol) in 25 ml DMF/CH_2Cl_2 (1:1) was stirred at 0°C and Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester (2.02 g, 2.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (0.44 g, 2.2 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.38 g, 2.7 mmol) dissolved in 10 ml DMF. The mixture was stirred for 96 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo*. The material was dissolved in CH_2Cl_2, washed with 1N HCl (3x100 ml), 5% $NaHCO_3$ (3x100 ml), and saturated NaCl solution (100 ml), respectively. Then the organic phase was dried over anhydrous Na_2SO_4 and removed *in vacuo*. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Nicotinyl-Ala-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester (1.82 g, 71.37%). TLC R_f=0.55, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1276, $(M+H)^+$.

Synthesis of Trigonellyl-Ala-Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester

Nicotinyl-Ala-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester (0.64g, 5.0 mmol) in 25 ml CH_2Cl_2 was stirred at 0°C, dimethylsulfate (0.32 g, 25 mmol) was added. The mixture was stirred for overnight at room temperature. The solvent then was removed at reduced pressure. The product was dissolved in CH_3OH and precipitated from ethyl ether to afford a white solid -- Trigonellyl-Ala-Ala-

Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester (0.58 g, 89.92%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1290, M^+.

Synthesis of Trigonellyl-Ala-Ala-Tyr-Arg-Cholesteryl Ester

Trigonellyl-Ala-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester (0.26 g, 0.20 mmol) was dissolved in 25 ml TFA/H_2O (19:1) and was stirred at 0°C for an hour. Then the solvent was removed at reduced pressure and the product was triturated with Et_2O three times and was recrystallized from Et_2O to afford a white solid -- Trigonellyl-Ala-Ala-Tyr-L-Arg-Cholesteryl Ester (0.17 g, 87.90%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=967, M^+.

Synthesis of Nicotinyl-Pro-Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester

Nicotinyl-Pro-OH (0.44g, 2.0 mmol) in 25 ml DMF/CH_2Cl_2 (1:1) was stirred at 0°C and Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester (2.20 g, 2.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (0.44 g, 2.2 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.38 g, 2.7 mmol) dissolved in 10 ml DMF. The mixture was stirred for 96 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo*. The material was dissolved in CH_2Cl_2, washed with 1N HCl (3x100 ml), 5% $NaHCO_3$ (3x100 ml), and saturated NaCl solution (100 ml), respectively. Then the organic phase was dried over anhydrous Na_2SO_4 and removed *in vacuo*. Silica-gel column

chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Nicotinyl-Pro-Ala-Tyr(O-tBu)-Arg(Pmc)-Cholesteryl Ester (1.31 g, 51.17%). TLC R_f=0.49, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1276, $(M+H)^+$.

Synthesis of KAYK-CDSs

Synthesis of Lys(Boc)-Cholesteryl Ester (1)

N-*a*-Fmoc-Lysine(Boc)-OH (4.68 g, 10.0 mmol) dissolved in 50 ml CH_2Cl_2 was stirred at 0°C and cholesterol (3.88 g, 10.0 mmol) dissolved in 75 ml CH_2Cl_2 was added, which was followed immediately by DCC (2.44 g, 11.0 mmol) dissolved in 25 ml CH_2Cl_2 and DMAP (1.68 g, 14.0 mmol) dissolved in 25 ml CH_2Cl_2 . The mixture was stirred for 48 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo* to give a solid -- N-*a*-Fmoc-Lys(Boc)-Cholesteryl Ester. The product was dissolved in 60 ml Piperidine/CH_2Cl_2 (1:3) and was stirred at room temperature for half an hour. Then the solvent was removed *in vacuo*. The crude material was dissolved in $CHCl_3$, washed with distilled water three times, and dried over anhydrous Na_2SO_4. Silica-gel column chromatography (7% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Lys(Boc)-Cholesteryl Ester (4.71 g, 76.4%). TLC R_f=0.65, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=637, $(M+Na)^+$. M.P.: 79~80°C. Elemental Analysis: Theory: H 10.82, C 74.22, N 4.56. Found: H 10.67, C 73.95, N 4.77.

Synthesis of Tyr-Lys(Boc)-Cholesteryl Ester (2)

Lys(Boc)-Cholesteryl Ester (3.08 g, 5.0 mmol) in 35 ml CH_2Cl_2 was stirred at 0°C and Fmoc-Tyr-OH (2.02 g, 5.0 mmol) dissolved in 35 ml CH_2Cl_2 was added, which was followed immediately by DCC (1.22 g, 5.5 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.91 g, 6.75 mmol) dissolved in 15 ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo* to give a solid -- N-*a*-Fmoc-Tyr-Lys(Boc)-Cholesteryl Ester. The peptide ester was deprotected with piperidine/CH_2Cl_2 in an identical manner illustrated in the preceding step. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid --Tyr-Lys(Boc)-Cholesteryl Ester (2.81 g, 72.14%). TLC R_f=0.58, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=801, $(M+Na)^+$. M.P.: 103 ~ 104°C. Elemental Analysis: Theory: H 9.72, C 72.55, N 5.40; Found: H 9.66, C 72.41, N 5.35.

Synthesis of Nicotinyl-Pro-OH (3)

Pro-OtBu ester (1.70 g, 10.0 mmol) in 25 ml CH_2Cl_2 was stirred at 0°C and then nicotinic acid (1.85 g, 15.0 mmol) dissolved in 25 ml DMF was added, which was followed immediately by DCC (2.42 g, 11.0 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (1.82 g, 13.0 mmol) dissolved in 25 ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU yielded was filtered off and the solvent

was removed *in vacuo*. The material obtained was dissolved in CH_2Cl_2 and washed with 1N HCl (3x100 ml), 5% $NaHCO_3$ (3x100 ml), and saturated NaCl solution (100 ml), respectively. Then the organic phase was dried over anhydrous Na_2SO_4 and evaporated to give a solid -- Nicotinyl-Pro-OtBu ester. The product was dissolved in 50 ml 95% TFA in H_2O and stirred for one hour at room temperature. Then the majority of the solvent was removed and the material was precipitated from ethyl ether to give a white solid -- Nicotinyl-Pro-OH (1.37 g, 62.29%). TLC R_f=0.12, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=221, $(M+H)^+$. M.P.: 176~177°C. Elemental Analysis: Theory: H 5.49, C 59.99, N 12.72; Found: H 5.50, C 60.08, N 12.66.

Synthesis of Nicotinyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (4)

Nicotinyl-Pro-OH (0.44g, 2.0 mmol) in 25 ml DMF/CH_2Cl_2 (1:1) was stirred at 0°C and Tyr-Lys(Boc)-Cholesteryl Ester (1.56 g, 2.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (0.44 g, 2.2 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.38 g, 2.7 mmol) dissolved in 10 ml DMF. The mixture was stirred for 96 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo*. The material obtained was dissolved in CH_2Cl_2 and was washed with 1N HCl (3x100 ml), 5% $NaHCO_3$ (3x100 ml), and saturated NaCl solution (100 ml), respectively. Then the organic phase was dried over anhydrous Na_2SO_4 and removed *in vacuo*. Silica-gel column

chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Nicotinyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (1.32 g, 67.28%). TLC R_f=0.47, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=981, $(M+H)^+$. M.P.: 121~123 °C. Elemental Analysis for Nicotinyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester•H_2O

Theory: H 8.78, C 69.78, N 7.01. Found: H 8.81, C 70.12, N 7.05.

Synthesis of Trigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (5)

Nicotinyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (0.49g, 5.0 mmol) in 50 ml ethyl ether was stirred at 0°C and dimethylsulfate (0.32 g, 25 mmol) was added. The mixture was stirred for overnight at room temperature. The product yielded was filtered off and washed with ethyl ether several times to afford a white solid -- Trigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (0.45 g, 91.84%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=996, M^+. M.P.: 153~155°C. Elemental Analysis for Trigonellyl$^+$-Pro-Tyr-Lys(Boc)-Cholesteryl Ester•HSO_4^- •H_2O: Theory: H 8.26, C 63.82, N 6.31. Found: H 8.17, C 63.69, N 6.19.

Synthesis of 1,4-Dihydrotrigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (6)

Trigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (0.2g, 2.0 mmol) in 15 ml CH_3OH/H_2O (1:1) was stirred at 0°C under argon. $NaHCO_3$ (0.17 g, 20 mmol) and $Na_2S_2O_4$ (0.35 g, 20 mmol) was then added little by little. The mixture was stirred at 0°C under argon for an hour. Then 35 ml saturated NaCl solution was added to dilute the solution which was extracted with 20 ml CH_2Cl_2. The organic

phase was dried over anhydrous Na_2SO_4 and evaporated. Natural aluminum oxide column chromatography (5% CH_3OH in CH_2Cl_2) afforded a yellow solid -- 1,4-Dihydrotrigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (0.05 g, 25.0%). UV spectrum showed the typical absorption peak for dihydro-compounds at 350 nm. TLC R_f=0.33, CH_3OH/CH_2Cl_2 (1:9). M.P.: decomposed. Elemental Analysis for 1,4-Dihydrotrigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester•$2H_2O$: Theory: H 9.08, C 68.64, N 6.78; Found: H 9.31, C 68.33, N 6.46.

Synthesis of Pro-Tyr-Lys(Boc)-Cholesteryl Ester (7)

Tyr-Lys(Boc)-Cholesteryl ester (3.89 g, 5.0 mmol) in 35 ml CH_2Cl_2 was stirred at 0°C and Fmoc-Pro-OH (1.69 g, 5.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (1.22 g, 5.5 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.91 g, 6.75 mmol) dissolved in 15 ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo* to give a solid -- N-*a*-Fmoc-Pro-Tyr-Lys(Boc)-Cholesteryl Ester. The peptide ester was deprotected in 35 ml Piperidine/ CH_2Cl_2 in an identical manner illustrated in the previous step. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Pro-Tyr-Lys(Boc)-Cholesteryl Ester (2.55 g, 58.22%). TLC R_f=0.51, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=898 $(M+Na)^+$. M.P.: 97~99°C. Elemental Analysis for Pro-Tyr-Lys(Boc)-Cholesteryl Ester•H_2O: Theory: H 9.48, C 69.92, N 6.27; Found: H 9.49, C 69.78, N 6.26.

Synthesis of Nicotinyl-Pro-Pro-Tyr-Lys-(Boc)Cholesteryl Ester (8)

Nicotinyl-Pro-OH (0.44g, 2.0 mmol) in 25 ml DMF/CH_2Cl_2 (1:1) was stirred at 0°C and Pro-Tyr-Lys(Boc)-Cholesteryl Ester (1.75 g, 2.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (0.44 g, 2.2 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.38 g, 2.7 mmol) dissolved in 10 ml DMF. The mixture was stirred for 96 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo*. The material obtained was dissolved in CH_2Cl_2 and washed with 1N HCl (3x100 ml), 5% $NaHCO_3$ (3x100 ml), and saturated NaCl solution (100 ml), respectively. Then the organic phase was dried over anhydrous Na_2SO_4 and evaporated. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Nicotinyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (1.41 g, 65.34%). TLC R_f=0.47, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1079, $(M+H)^+$. M.P.: 131~132°C. Elemental Analysis: Theory: H 8.61, C 70.23, N 7.80. Found: H 8.62, C 69.84, N 7.68.

Synthesis of Trigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (9)

Nicotinyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (0.51g, 5.0 mmol) in 50 ml ethyl ether was stirred at 0°C and dimethylsulfate (0.32 g, 25 mmol) was added. The mixture was stirred for overnight at room temperature. The product

yielded was filtered off and washed with ethyl ether several times to afford a white solid -- Trigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (0.48 g, 87.75%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1094, M^+. M.P.: 169~170°C. Elemental Analysis for Trigonellyl$^+$-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester•HSO_4^- •H_2O: Theory: H 8.25, C 63.91, N 6.88, Found: H 8.25, C 63.95, N 6.91.

Synthesis of 1,4-Dihydrotrigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (10)

Trigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (0.22 g, 2.0 mmol) in 15 ml CH_3OH/H_2O (1:1) was stirred at 0°C under argon and $NaHCO_3$ (0.17 g, 20 mmol) was added, which was followed by $Na_2S_2O_4$ (0.35 g, 20 mmol) added little by little. The mixture was stirred at 0°C under argon for an hour. Then 35 ml saturated NaCl solution was added to dilute the solution and 25 ml CH_2Cl_2 was added to extract the product. The organic phase was dried over anhydrous Na_2SO_4 and evaporated. Natural aluminum oxide column chromatography (5% CH_3OH in CH_2Cl_2) afforded a yellow solid -- 1,4-Dihydrotrigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (0.06 g, 27.3%). UV spectrum showed the typical absorption peak for dihydro-compounds at 350 nm. Yield: %. TLC R_f=0.35, CH_3OH/CH_2Cl_2 (3%). M.P.: decomposed. Elemental Analysis for 1,4-Dihydrotrigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester•$3H_2O$: Theory: H 8.96, C 66.99, N 7.32; Found: H 9.08, C 66.87, N 7.48.

Synthesis of Ala-Tyr-Lys(Boc)-Cholesteryl Ester (11)

Tyr-Lys(Boc)-Cholesteryl ester (3.89 g, 5.0 mmol) in 35 ml CH_2Cl_2 was stirred at 0°C and Fmoc-Ala-OH (1.53 g, 5.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (1.22 g, 5.5 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.91 g, 6.75 mmol) dissolved in 15 ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo* to give a solid -- N-*a*-Fmoc-Ala-Tyr-Lys(Boc)-Cholesteryl Ester. The peptide ester was deprotected in 35 ml Piperidine/ CH_2Cl_2 in an identical manner illustrated in the previous step. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Ala-Tyr-Lys(Boc)-Cholesteryl Ester (3.11 g, 73.0%). TLC R_f=0.50, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=872, $(M+Na)^+$. M.P.: 103~104 °C. Elemental Analysis: Theory: H 9.41, C 70.59, N 6.59. Found: H 9.49, C 70.43, N 6.59.

Synthesis of Nicotinyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester (12)

Nicotinyl-Pro-OH (0.44g, 2.0 mmol) in 25 ml DMF/CH_2Cl_2 (1:1) was stirred at 0°C and Ala-Tyr-Lys(Boc)-Cholesteryl Ester (1.70 g, 2.0 mmol) dissolved in 25 ml CH_2Cl_2 was added, which was followed immediately by DCC (0.44 g, 2.2 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.38 g, 2.7 mmol) dissolved in 10 ml DMF. The mixture was stirred for 96 hours at room temperature.

The DCU yielded was filtered off and the solvent was removed *in vacuo*. The material obtained was dissolved in CH_2Cl_2 and was washed with 1N HCl (3x100 ml), 5% $NaHCO_3$ (3x100 ml), and saturated NaCl solution (100 ml), respectively. Then the organic phase was dried over anhydrous Na_2SO_4 and removed *in vacuo*. Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Nicotinyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester (1.53 g, 72.65%). TLC R_f=0.46, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1053 $(M+H)^+$. M.P.: 155~156°C. Elemental Analysis: Theory: H 8.63, C 69.68, N 7.99; Found: H 8.68, C 69.46, N 7.96.

Synthesis of Trigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester (13)

Nicotinyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester (0.51g, 5.0 mmol) in 50 ml ethyl ether with a few drops of MeOH was stirred at 0°C and dimethylsulfate (0.32 g, 25 mmol) was added. The mixture was stirred for overnight at room temperature. The product yielded was filtered off and washed with ethyl ether several times to afford a white solid -- Trigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester (0.49 g, 91.76%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=1068, M^+. M.P.: 185~187°C. Elemental Analysis for Trigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester•HSO_4^-•$2H_2O$: Theory: H 8.23, C 62.08, N 7.01; Found: H 8.29, C 62.22, N 6.75.

Synthesis of 1,4-Dihydrotrigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester (14)

Trigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester (0.21 g, 2.0 mmol) in 15 ml CH_3OH/H_2O (1:1) was stirred at 0°C under argon. $NaHCO_3$ (0.17 g, 20 mmol) and $Na_2S_2O_4$ (0.35 g, 20 mmol) were added little by little. The mixture was stirred at 0°C under argon for an hour. Then 35 ml saturated NaCl solution was added to dilute the solution which was then extracted with 25 ml CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4 and evaporated. Natural aluminum oxide column chromatography (5% CH_3OH in CH_2Cl_2) afforded a yellow solid -- 1,4-Dihydrotrigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester (0.06 g, 28.6%). UV spectrum showed the typical absorption peak for dihydro-compounds at 350 nm. TLC R_f= 0.38, CH_3OH/CH_2Cl_2 (3%). M.P.: decomposed. Elemental Analysis for 1,4-Dihydrotrigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester•$3H_2O$: Theory: H 8.99 C 66.40 N 7.49; Found: H 9.01, C 66.60, N 7.47.

Synthesis of BTRA

Synthesis of Lys(Fmoc)-Cholesteryl Ester (15)

N-*a*-Boc-Lysine(Fmoc)-OH (4.68 g, 10.0 mmol) in 75 ml CH_2Cl_2 was stirred at 0°C and cholesterol (3.88 g, 10.0 mmol) dissolved in 75 ml CH_2Cl_2 was added, which was followed immediately by DCC (2.44 g, 11.0 mmol) dissolved in 15 ml CH_2Cl_2 and DMAP (1.68 g, 14.0 mmol) dissolved in 25 ml CH_2Cl_2 . The

mixture was stirred for 48 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo* to give a solid -- N-*a*-Boc- Lys(Fmoc)-Cholesteryl Ester. The peptide ester was deprotected in 60 ml TFA/CH_2Cl_2 (1:1) for an hour at the room temperature and the solvent was then removed at reduced pressure. Silica-gel column chromatography (7% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid -- Lys(Boc)-Cholesteryl Ester (4.31 g, 58.48%). TLC R_f=0.35, CH_3OH/CH_2Cl_2 (1:19). Mass spectrum: m/z=760, $(M+Na)^+$. M.P.: 74~75°C. Elemental Analysis: Theory: H 9.30, C 78.22, N 3.80. Found: H 9.36, C 78.14, N 3.85.

Synthesis of Boc-Tyr-Lys-Cholesteryl Ester (16)

Lys(Fmoc)-Cholesteryl ester (3.69 g, 5.0 mmol) in 35 ml CH_2Cl_2 was stirred at 0°C and Boc-Tyr-OH (1.41 g, 5.0 mmol) dissolved in 35 ml CH_2Cl_2 was added, which was followed immediately by DCC (1.22 g, 5.5 mmol) dissolved in 15 ml CH_2Cl_2 and HOBt (0.91 g, 6.75 mmol) dissolved in 15 ml DMF. The mixture was stirred for 24 hours at room temperature. The DCU yielded was filtered off and the solvent was removed *in vacuo* to give a solid -- N-*a*-Boc-Tyr-Lys(Fmoc)-Cholesteryl Ester. The peptide ester was deprotected in 50 ml piperidine/CH_2Cl_2 (1:3) in an identical manner illustrated in the previous step. Silica-gel column chromatography (3% AcOH and 10% CH_3OH in CH_2Cl_2) afforded a white solid -- Boc-Tyr-Lys-Cholesteryl Ester (3.19 g, 81.90%). TLC R_f=0.05, CH_3OH/CH_2Cl_2 (1:10). Mass

spectrum: m/z=801, $(M+Na)^+$. M.P.: 121 ~ 123°C. Elemental Analysis: Theory: H 9.72, C 72.55, N 5.40. Found: H 9.69, C 72.44, N 5.40.

Synthesis of N-(2,4-Dinitrophenyl)-Nicotinamide Chloride (17){PRIVATE }

Nicotinamide (2.5 g, 20 mmol) was mixed with 1-chloro-2,4-dinitrophenyl chloride (6.25 g, 30 mmol) in 20 ml DMF and gradually heated to 100°C. The mixture was stirred for one hour at that temperature and then cooled down to the room temperature. The material obtained was dissolved in MeOH (30 ml) and poured into 125 ml vigorously stirred Et_2O. The precipitant was again dissolved in 30 ml MeOH and poured into 125 ml Et_2O. The precipitant was then dissolved in 50 ml MeOH and stirred with active carbon for 30 minutes. The active carbon was filtered off and the filtrate was evaporated *in vacuo* to give the title compound (2.69 g, 46.54%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:10). Mass spectrum: m/z=289, M^+. M.P.: 177 ~ 179°C. Elemental Analysis: Theory: H 2.77, C 44.38, N 17.16; Found: H 2.71, C 44.69, N 17.29.

Synthesis of Boc-Tyr-Nys$^{\pm}$-Cholesteryl Ester (18)

Boc-Tyr-Lys-Cholesteryl Ester (1.56g, 2.0 mmol) in CH_3OH was stirred at 0°C and N-(2,4-dinitrophenyl)-nicotinamide chloride (0.65 g, 2.25 mmol) was added. The mixture was stirred for overnight at room temperature. The product was precipitated from ethyl ether, filtered off, and washed with ethyl ether several

times. Sephadex LH-20 column chromatography (CH_3OH) afforded a yellow solid -- Boc-Tyr-Nys$^+$-Cholesteryl Ester (1.45 g, 81.92%). TLC R_f=0, CH_3OH/CH_2Cl_2 (1:9). Mass spectrum: m/z=885, M^+. M.P.: 168~169°C. Elemental Analysis for Boc-Tyr-Nys$^+$-Cholesteryl Ester Cl^- •$3H_2O$: Theory: H 8.89, C 65.31, N 5.75; Found: H 8.82, C 64.94, N 5.68.

Synthesis of Boc-Tyr-Nys-Cholesteryl Ester (19)

Boc-Tyr-Nys$^+$-Cholesteryl Ester (0.18g, 2.0 mmol) in 15 ml CH_3OH/H_2O (1:1) was stirred at 0°C under argon. $NaHCO_3$ (0.17 g, 20 mmol) and $Na_2S_2O_4$ (0.35 g, 20 mmol) was added little by little. The mixture was stirred at 0°C under argon for an hour. Then 35 ml saturated NaCl solution was added to dilute the solution which was then extracted with 25 ml CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4 and was removed *in vacuo*. Natural aluminum oxide column chromatography (5% CH_3OH in CH_2Cl_2) afforded a yellow solid -- Boc-Tyr-Nys-Cholesteryl Ester (0.05g, 27.8%). UV spectrum showed the typical absorption peak for dihydro-compounds at 350 nm. TLC R_f=0.33 CH_3OH/CH_2Cl_2 (3%).
M.P.: decomposed. Elemental Analysis for Boc-Tyr-Nys-Cholesteryl Ester•H_2O: Theory: H 9.15, C 70.48, N 6.20; Found: H9.25, C 70.60, N 6.17.

CHAPTER 4
RESULTS AND DISCUSSION

Peptide Synthesis -- The DCC/HOBt Method

The coupling method used in all the syntheses was the DCC/HOBt method. The carbodiimide method (Khorana, 1953) was introduced to peptide synthesis in 1955 (Sheehan & Hess, 1955). This method uses DCC (dicyclohexylcarbodiimide) to achieve dehydration and peptide bond formation. DCU (*N,N'*-Dicyclohexylurea) is formed as a by-product. The DCC method in its original form is accompanied by side reactions. Activation of amino acids or peptide acids with DCC, especially in the presence of base, yields *N*-acylureas which decrease yields and frequently complicate products. Considerable racemization is also observed (Anderson & Callahan, 1958). These problems stimulated studies to determine mechanisms of carboxyl activation and acyl-peptide racemization and also led to the practice of adding nucleophiles to DCC reaction mixtures to suppress side-reactions. HOBt (hydroxybenzotriazole) is probably the best neucleophile for such a purpose and is highly effective in suppressing racemization and *N*-acylurea formation under certain conditions -- such as mildly basic condition and low dielectric constant medium (Fig. 20). The DCC/HOBt combination is an extremely

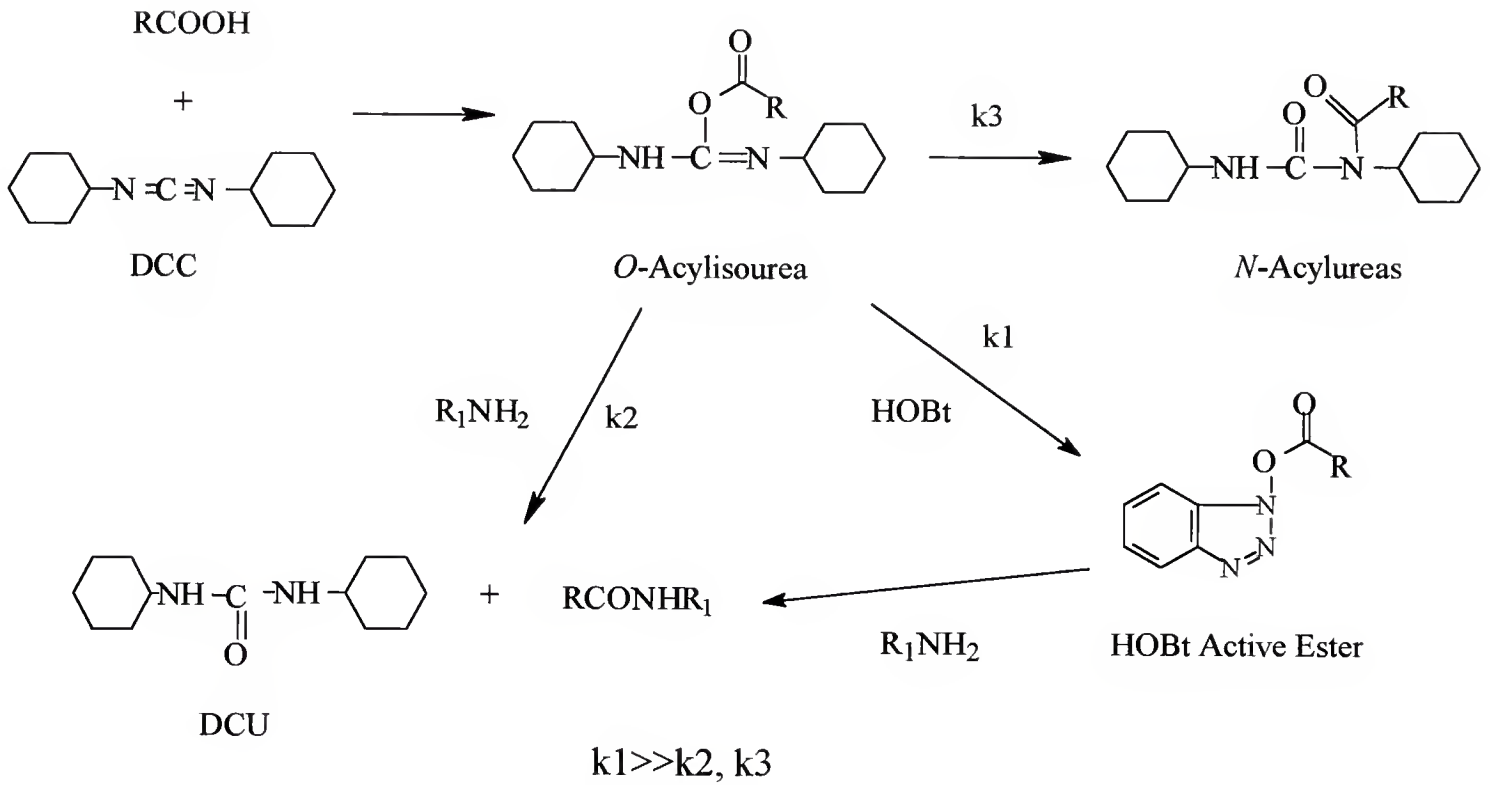


Figure 20. The Mechanism of the DCC/HOBt Method

efficient method for coupling peptides in solution. HOBt apparently reacts so rapidly with *O*-acylisourea that the competing intramolecular reaction (*N*-acylurea formation) is not observed (Konig & Geiger, 1970). The DCC/HOBt combination also suppresses other side reactions. The use of the DCC/HOBt coupling method in the synthesis of brain-targeted delivery systems for Kyotorphin and KAYK was successful.

Synthesis of Kyotorphin CDS's

Table 5 lists the synthesized intermediates for Kyotorphin-CDS by using the Boc method and Table 6 lists the synthesized intermediates for Kyotorphin-CDS by using the Fmoc method.

Table 5. The Intermediates for the Kyotorphin-CDS Synthesized by the Boc Method

Compound Name	**Abb.**	**MW**
Arg(NO_2)-Cholesteryl Ester	R(NO_2)C	588
Tyr-Arg(NO_2)-Cholesteryl Ester	YR(NO_2)C	751
Nicotinyl-Ala-OH	NA-OH	194
Nicotinyl-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester	NAYR(NO_2)C	927
Trigonellyl-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester	TAYR(NO_2)C	952
Ala-Tyr-Arg(NO_2)-Cholesteryl Ester	AYR(NO_2)C	822
Nicotinyl-Ala-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester	NAAYR(NO_2)C	998

Table 6. The Intermediates and a Kyotorphin-CDS Synthesized by the Fmoc Method

Compound Name	**Abb.**	**MW**
Arg(Pmc)-Cholesteryl Ester	R(P)C	809
Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	Y(B)R(P)C	1029
NP-OH	NP-OH	220
Nicotinyl-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	NAY(B)R(P)C	1205
Trigonellyl-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	TAY(B)R(P)C	1220
Trigonellyl-Ala-Tyr-Arg-Cholesteryl Ester	TAYRC	896
1,4-Dihydrotrigonellyl-Ala-Tyr-Arg-Cholesteryl Ester	DAYRC	897
Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	AY(B)R(P)C	1100
Nicotinyl-Ala-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	NAAY(B)R(P)C	1276
Trigonellyl-Ala-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	TAAY(B)R(P)C	1291
Trigonellyl-Ala-Ala-Tyr-Arg-Cholesteryl Ester	TAAYRC	967
Nicotinyl-Pro-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	NPY(B)R(P)C	1231
Trigonellyl-Pro-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	TPY(B)R(P)C	1246
Nicotinyl-Pro-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	NPAY(B)R(P)C	1302
Trigonellyl-Pro-Ala-Tyr(OtBu)-Arg(Pmc)-Cholesteryl Ester	TPAY(B)R(P)C	1317

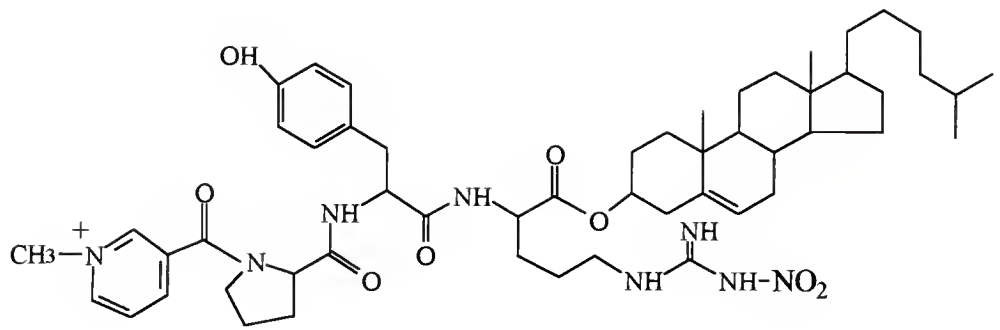
Although kyotorphin is only a dipeptide, both arginine and tyrosine have side chain groups that may be protected to achieve a smooth synthesis. Both the Boc and the Fmoc methods were employed during the synthesis of chemical delivery systems for kyotorphin.

As shown in Table 5 and Table 6, the synthesis of Kyotorphin-CDS's was not completed.

Synthesis of Kyotorphin-CDS's by Using the Boc Method

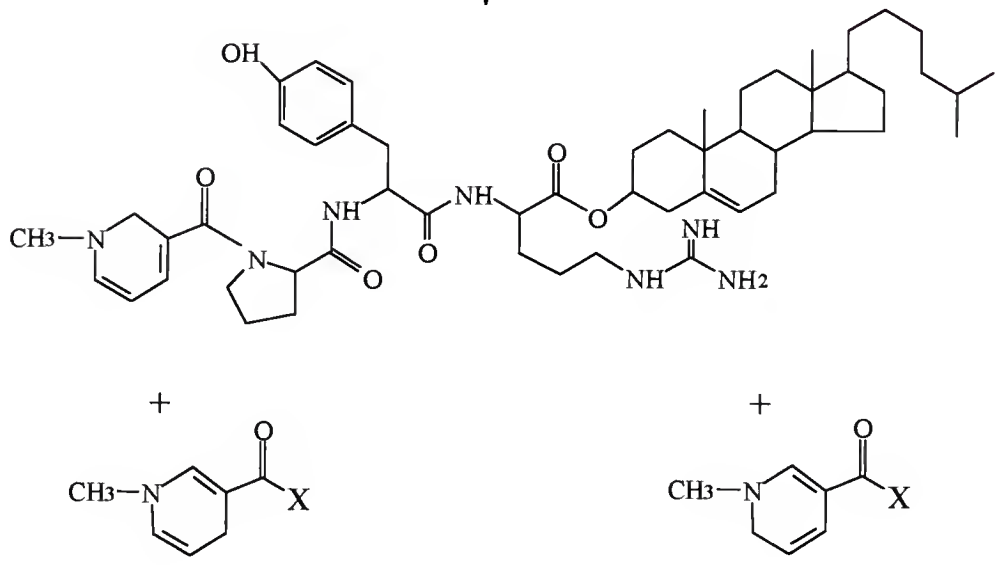
A sufficient amount of Trigonellyl-Ala-Tyr-Arg(NO_2)-Cholesteryl Ester has been synthesized (2 grams) by using the Boc method. Difficulties were encountered at the removal of the side-chain protection group (ε-NO_2) of arginine. Two deprotection methods were attempted: acidolysis with strong acids and hydrogenesis. The problem is that the ester bond between cholesterol and arginine is unstable at severe acidolysis conditions, such as high concentration of HF, Boron tris (trifluoroacetate), and TFA/TMSOTf. Hydrogenolysis, on the other hand, keeps the ester bond intact but reduces the pyridine ring of the trigonellyl group that is essential to the CDS (Figure 21). This route was then given up in favor of others.

The guanine group of arginine is very basic (pKa $\sim$ 12.5) so that it is inert in liquid-phase synthesis except in extreme basic environment. Therefore, it is possible to synthesize Kyotorphin-CDS's while leaving the side-chain group unprotected. However, the N-α-Boc-Arg•HCl is very hydrophilic and the



Trigonellyl-Pro-Tyr-Arg(NO_2)-Cholesteryl Ester

Hydrogenolysis



Dihydrotrigonellyl-Pro-Tyr-Arg-Cholesteryl Esters

Figure 21. Removal of the ε-NO_2 group of a Kyotorphin-CDS by Hydrogenolysis

cholesterol is very lipophilic. Problems exist during the coupling reaction: 1) the solubility of N-*a*-Boc-Arg•HCl is low even in DMF; 2) The rate of the coupling reaction is slower than those of the side-chain protected methods, and the long reaction time results in more by-products; and 3) The unprotected guanine group of arginine is still sensitive to the Me_2SO_4 quarternization as indicated by the quarternization of Boc-Arg-Cholesteryl Ester. Therefore, the use of side-chain unprotected arginine to synthesize Kyotorphin-CDS is not feasible.

Synthesis of Kyotorphin-CDS's by Using the Fmoc Method

N-α-Fmoc amino acids are seldom used in liquid-phase synthesis because their deprotection by-products, dibenzofulvene and their derived polymer or amine abducts, are difficult to separate from the products. However, in the synthesis of Kyotorphin-CDS, the Fmoc method showed its advantage: Side-chain protection is possible as the protecting groups can be easily removed under relatively mild acidic conditions with TFA (trifluoro acetic acid) which keeps the cholesterol-arginine ester bond intact. Synthesis with protected side-chain makes the synthesis relatively smooth and easy.

One of the Kyotorphin-CDS -- 1,4-Dihydrotrigonellyl-Ala-Tyr-Arg-Cholesteryl Ester has been synthesized. However, while the basicity (pKa ~ 12.5) of the guanine group of the arginine makes Kyotorphin-CDS difficult to synthesize, the problem is further complicated by its positive charge status *in vivo* that reduces

the efficiency of Kyotorphin-CDS to penetrate the BBB. Therefore, a protection group to protect the guanine group would be desired to ensure the desired lipophilicity of Kyotorphin-CDS. But, the addition of a protection group to the ε-position of arginine of Kyotorphin-CDS is not without problems:

1. Since the guanine group is inert in liquid phase except under extreme basic conditions, the addition of a protection group is very difficult. Bases like triethylamine or DIEA are not strong enough to expose the ε-NH_2 group of arginine while stronger bases, such as NaOH, present problems to the delicate molecular package of Kyotorphin-CDS.

2. Another potential problem is that the basic nitrogen on the pyridine ring of nicotinic acid and the hydroxy group of tyrosine may compete with the guanine group and yield by-products.

Nevertheless, attempts were made to add a labile protection group to the ε-NH_2 group of arginine (Fig. 22).

Synthesis was attempted according to Figure 22 in the presence of Et_3N or DIEA, respectively. Three attempts were made for each base and all attempts failed.

Literature research revealed that the synthesis of ε-substituted/protected arginine was most frequently accomplished by using nucleophilic attack by the ε-amino group of ornithine on the methylthiocarbonyl of a

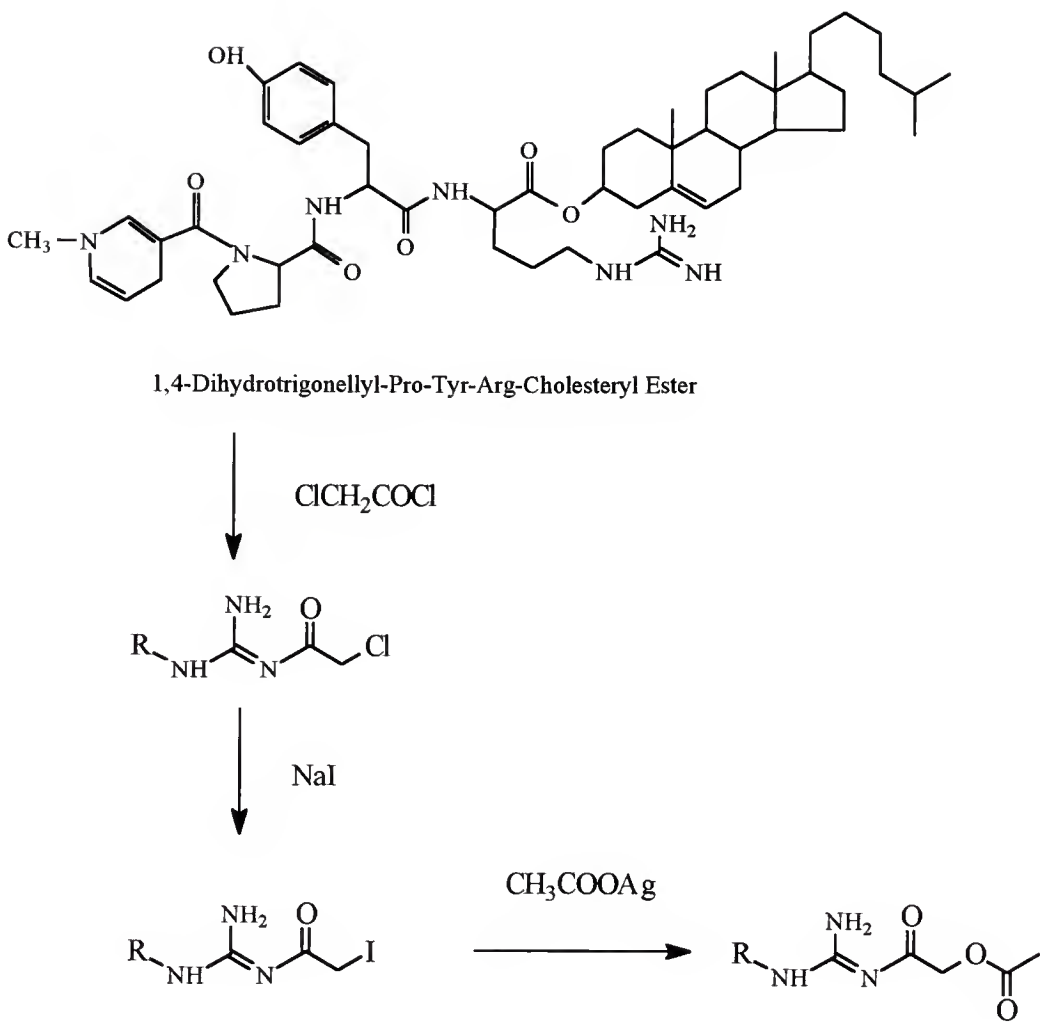


Figure 22. The Proposed Synthetic Scheme for the Addition of a Labile Protection Group to ε-NH_2 Group of Arginine

substituted pseudothiouronium salt, thus avoiding dealing with the inert guanine group (Cho *et al.* 1984; Olken & Marletta, 1992; Wallace & Fukuto, 1991). However, the method is not applicable to Kyotorphin-CDS's due to the fact that our proposed protection group is not stable enough to go through the multi-step synthesis. Considering the high cost of Nicotinyl-Ala-Tyr-Arg-Cholesteryl Ester, even if a method can be found to protect the guanine group, the destined low yield (due to the inertness of the guanine group and the presence of other active function groups) would make the process too expensive. After all the considerations, Kyotorphin-CDS was replaced by KAYK-CDS.

Synthesis of KAYK-CDS's and BTRA

Table 7 lists the synthesized intermediates and final products for KAYK-CDS's and BTRA.

The kyotorphin analog used for KAYK-CDS's was L-Tyr-L-Lys. The reason it was selected is that it exhibits almost the same ED_{50} and TD_{50} as kyotorphin (Rolka *et al.*, 1983).

There are several inherent advantages for using lysine to replace arginine:

1. The ε side-chain protection groups for lysine can be removed at mild conditions, so that it is possible to remove the ε side-chain protection groups of lysine without damaging the molecular package of the delivery systems.

Table 7. The Intermediates and KAYK-CDS's and BTRA Synthesized

Compound Name	**Abb.**	**MW**
Lys(Boc)-Cholesteryl Ester	K(B)C	615
Tyr-Lys(Boc)-Cholesteryl Ester	YK(B)C	779
NP-OH	NP-OH	220
Nicotinyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester	NPYK(B)C	981
Trigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester	TPYK(B)C	996
1,4-Dihydrotrigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester	CDS-P	997
Pro-Tyr-Lys(Boc)-Cholesteryl Ester	PYK(B)C	876
Nicotinyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester	NPPYK(B)C	1079
Trigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester	TPPYK(B)C	1094
1,4-Dihydrotrigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester	CDS-PP	1095
Ala-Tyr-Lys(Boc)-Cholesteryl Ester	AYK(B)C	850
Nicotinyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester	NPAYK(B)C	1053
Trigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester	TPAYK(B)C	1068
1,4-Dihydrotrigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester	CDS-PA	1069
Lys(Fmoc)-Cholesteryl Ester	K(F)C	737
Boc-Tyr-Lys-Cholesteryl Ester	BYKC	779
N-(2,4-Dinitrophenyl)-Nicotinamide Chloride	DNC	289
Boc-Tyr-Nys^{+}-Cholesteryl Ester	BYNC	885
Boc-Tyr-Nys-Cholesteryl Ester	BTRA	886

2. Both N-α-Fmoc-ε-Boc-Lysine and N-α-Boc-ε-Fmoc-Lysine are commercially available which simplifies the synthesis.

3. The Boc group can be hydrolyzed *in vivo*, so that the ε-Boc can be a part of the CDS molecular package that further simplifies the synthesis.

4. The ε-NH_2 group itself may be modified to function as the 1,4-dihydrotrigonellyl group, which leads to the Brain-Targeted Redox Analog (BTRA).

The use of DCC/HOBt coupling method with Fmoc- and Boc-amino acid derivatives in the syntheses of all three KAYK-CDS's and BTRA was successful. The optimal reaction conditions included an initial reaction temperature at 0°C and using solvents with as low as a dielectric constant as possible. No major side reaction was found during the entire synthesis.

The only problem encountered during the synthesis was in the synthesis of Trigonellyl-Pro-Tyr-Lys(Boc)-Cholesteryl Ester, Trigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester, and Trigonellyl-Pro-Ala-Tyr-Lys(Boc)-Cholesteryl Ester. The choice of solvent was proved to be critical. The use of CH_2Cl_2 or CH_3OH as solvent caused the premature loss of the ε-Boc group on lysine. It was possible duo to the formation of H_2SO_4 during the quarternization process. The use of Et_2O as solvent solved the problem. Trigonellyl compounds do not dissolve in Et_2O. Therefore, as soon as the trigonellyl compounds form during the reaction, they precipitate from the solvent and hence protect the Boc group from H_2SO_4 in the solvent.

Generally saying, the synthesis of KAYK-CDS's was relatively smooth compared to the synthesis of Kyotorphin-CDS's. As shown in Table 4, all the intermediates, KAYK-CDS's and BTRA have been successfully synthesized.

Pharmacology Studies of KAYK-CDS's and BTRA

Tail-flick response was used to determine the analgesic effects of KAYK-CDS's and BTRA. The method is simple to use and offers great stability in the pain threshold under a variety of conditions (D' Amour & Smith, 1941).

The dose-response relationship of one of the KAYK-CDS -- CDS-P is shown in Table 8 and Figure 23. the maximum response was defined as the mean of the maximum tail-flick latencies measured from each rat in each group, regardless of the time they were recorded.

CDS-P, at all doses tested, significantly increased rat tail-flick latency periods compared to vehicle. Dose dependent response was observed among dose levels of 0.003, 0.0074, and 0.0148 mmol/kg (equimolar to 1.0, 2.5, and 5.0 mg/kg of kyotorphin, respectively; single-factor anova for the maximum responses, $p<0.01$). However, there was no significant difference between dose levels of 0.0148 and 0.0223 mmol/kg (equimolar to 5.0 and 7.5 mg/kg of kyotorphin; single-factor anova for the maximum responses, $p=0.7598$).

The analgesic effects of KAYK-CDS's and BTRA were compared with those of vehicle and unmodified kyotorphin after i.v. administration at the dose of 0.0223

Table 8. The Rat Tail Flick Latency Periods Prior to and After i.v. Administration of Different Doses of CDS-P in Rats [a, b]

	The Rat Tail Flick Latency Period (sec.)				
Time	Vehicle	CDS-P 1.0 [c]	CDS-P 2.5 [c]	CDS-P 5.0 [c]	CDS-P 7.5 [c]
0 min	7.4±1.1	7.7±1.0	8.8±1.3	7.3±1.5	6.7±1.5
15 min	7.4±1.5	9.4±2.2	12.4±2.2	13.9±5.5	18.7±3.5
30 min	7.6±1.0	10.5±3.1	13.9±2.1	19.1±2.0	23.5±3.3
1 hr	7.0±1.0	10.7±3	18±1.2	22.6±3.9	22.1±5.0
2 hr	7.7±1.3	10.3±2.6	18.5±1	22.6±2.4	23.1±6.9
3 hr	8.6±2.1	10.3±2.3	18.6±1.1	23.7±3.6	21.6±5.0
4 hr	8.1±1.0	11±2.2	17.9±2.9	23.0±3.5	20.3±3.8
5 hr	8.9±2.3	11.2±2.7	17.9±3.4	19.6±3.4	19.4±1.9
6 hr	8.0±1.7	10.7±2.1	15.3±2.3	17.9±1.9	17.3±1.4
Maximum Responses	10.1±1.4	13.1±1.6	20.7±1.5	26.1±1.3	26.5±5.3
p-vehicle		<0.01	<0.01	<0.01	<0.01
p-CDS-P1.0	<0.01		<0.01	<0.01	<0.01
p-CDS-P2.5	<0.01	<0.01		<0.05	<0.01
p-CDS-P5.0	<0.01	<0.01	<0.05		0.7598
p-CDS-7.5	<0.01	<0.01	<0.01	0.7598	

[a] Data represents mean ± SD of 6 rats.
[b] p: single-factor anova
[c] equimolar dose to 1.0, 2.5, 5.0, 7.5 mg/kg of kyotorphin, respectively.

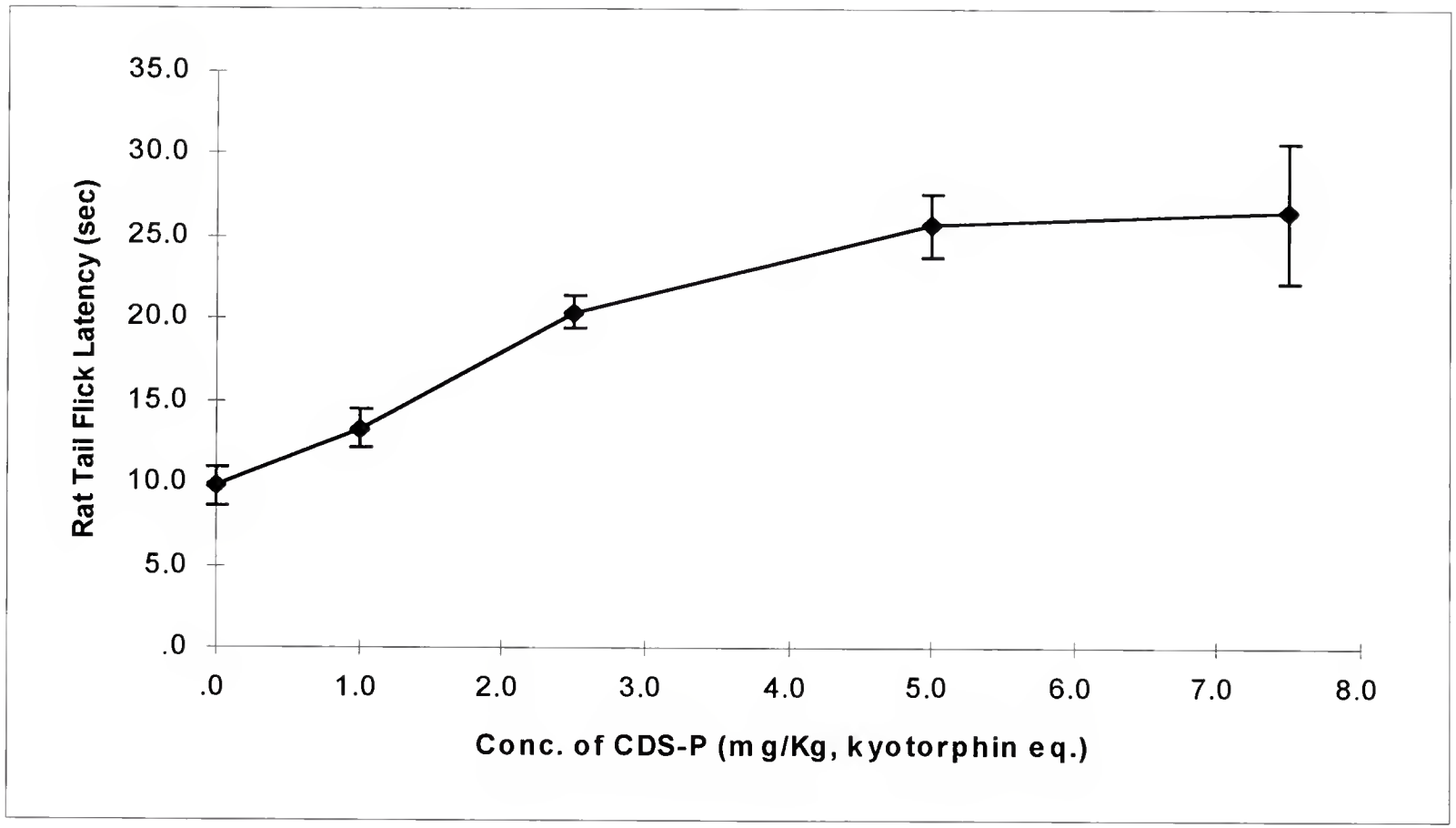


Figure 23. Dose Response of CDS-P after i.v. Administration in Rat

* Data represents mean ± SD of 6 rats.

mmol/kg (equimolar to 7.5 mg/kg of kyotorphin; Table 9 and Figure 24). The results indicated that all of the brain-targeted delivery systems for KAYK showed significant analgesia effects when compared with vehicle while kyotorphin did not. Among the four delivery systems, CDS-PP showed statistically significant higher analgesic effect than the others, followed by CDS-P and BTRA, and CDS-PA showed the lowest analgesic effect (single-factor anova for the maximum responses, $p<0.05$).

The results indicated that kyotorphin alone did not have any analgesia effect after i.v. administration at the dose of 7.5 mg/kg (Table 9; single-factor anova, $p=0.4821$), but all four KAYK brain-targeted delivery systems had analgesia effects at the equimolar dose.

The time-response relationships of the four KAYK brain delivery systems were also studied (Table 9, Table 10 and Figure 25). All the brain delivery systems displayed the desired sustained effects compared with the vehicle and unmodified kyotorphin (two-factor anova with replication, $p<0.05$). CDS-PP showed strongest analgesic effects across the six-hour period tested. Although there was no significant difference between the analgesic effects of CDS-P and BTRA at 15 minutes and 30 minutes after i.v. administration (two-factor anova with replication, $p=0.07$), CDS-P showed stronger analgesic effect after one hour (two-factor anova with replication, $p<0.05$). BTRA showed stronger analgesic effect over CDS-PA in the category of

Table 9. The Rat Tail Flick Latency Period Prior to and After i.v. Administration of KAYK-CDS's and BTRA [a, b, c]

	The Rat Tail Flick Latency Period (sec.)					
Time	Vehicle	KTP	CDS-PP	CDS-PA	BTRA	CDS-P
0 min	7.4±1.1	7.3±1.4	7.4±1.1	7.2±1.4	8.3±1.6	6.7±1.5
15 min	7.4±1.5	6.8±1.4	22.5±6.1	16.7±2.3	18.7±5.8	18.7±3.5
30 min	7.6±1.0	7.6±2.1	34.9±6.3	19.2±2.6	25.7±6.4	23.5±3.3
1 hr	7.0±1.0	8.5±2.1	31.8±4.5	18.6±2.3	18.5±2.5	22.1±5.0
2 hr	7.7±1.3	8.6±0.7	24.5±3.9	15.8±2	15.2±2.7	23.1±6.9
3 hr	8.6±2.1	8.8±2.3	25.1±3.9	15.7±1.4	15.8±2.3	21.6±5.0
4 hr	8.1±1.0	7.3±1.3	22.7±2.9	16±1.4	16±3.0	20.3±3.8
5 hr	8.9±2.3	7.6±2.5	23.2±3.5	16.3±1.4	15.1±1.3	19.4±1.9
6 hr	8.0±1.7	7.5±2.7	19.8±2.6	13.9±1.7	12.6±1.3	17.3±1.4
Maximum Responses	10.1±1.4	10.4±1.5	36.8±5.0	19.7±2.1	26.1±6.0	26.5±5.3
p-vehicle		0.4821	<0.01	<0.01	<0.01	<0.01
p-KTP	0.4821		<0.01	<0.01	<0.01	<0.01
p-CDS-PP	<0.01	<0.01		<0.01	0.01069	<0.01
P-CDS-PA	<0.01	<0.01	<0.01		0.03961	0.02003
p-BTRA	<0.01	<0.01	0.01069	0.03961		0.9220
p-CDS-P	<0.01	<0.01	<0.01	0.02003	0.9220	

[a] Data represents mean ± SD of 6 rats.

[b] One of the six rats from each of the groups of CDS-P and BTRA reached cut-off latency during the test and was eliminated.

[c] p: single-factor anova

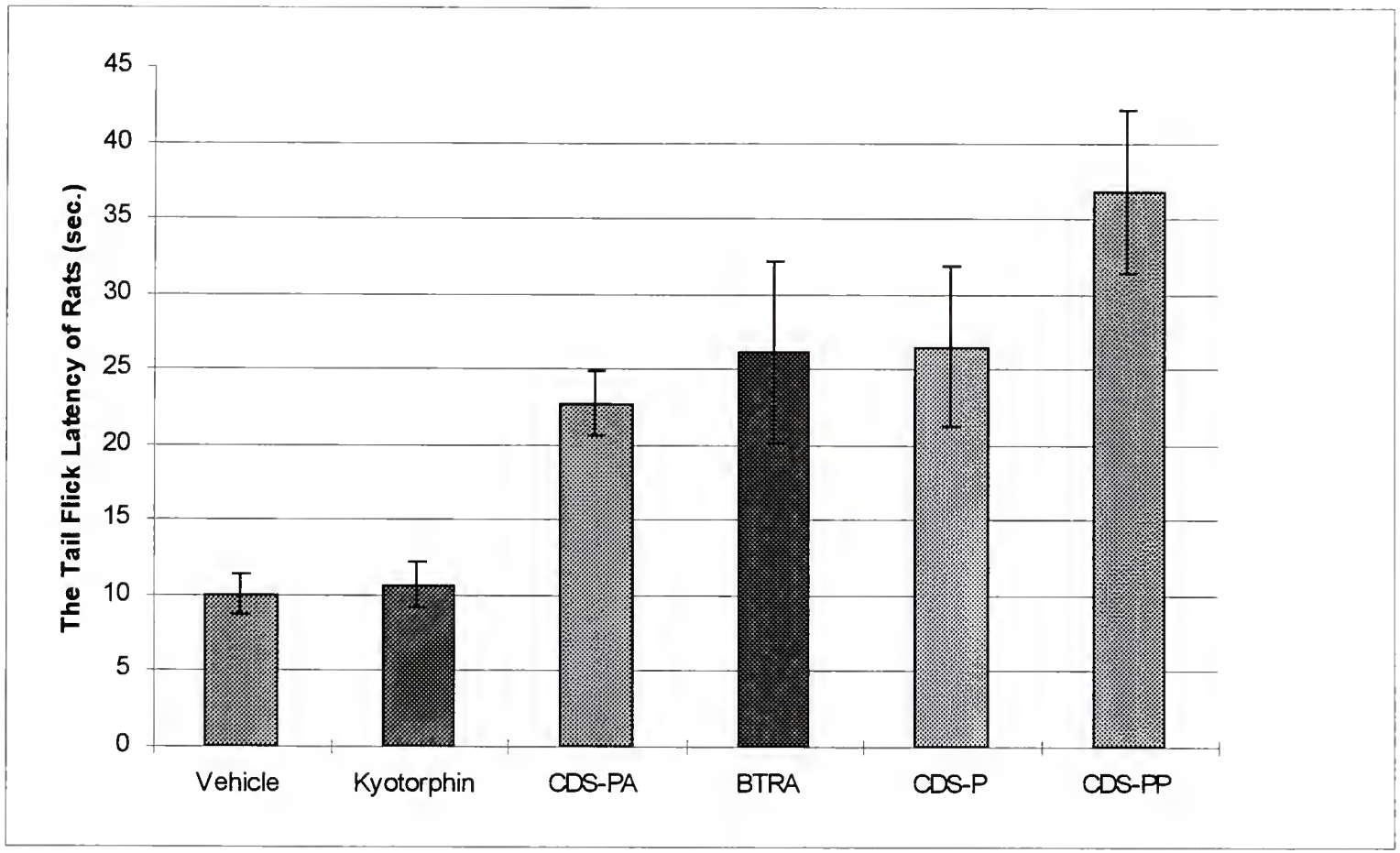


Figure 24. The Rat Tail-Flick Latency Periods of KAYK-CDS's and BTRA after i.v. Administration
* Data represents mean ± SD of 6 rats.

Table 10. Two-Factor Anova with Replication for Comparison of the Rat Tail Flick Latency Prior to and After i.v. Administration of KAYK-CDS's and BTRA over the Six Hour Testing Period

p-Vehicle vs KTP	0.83617
p-Vehicle vs CDS-PA	<0.01
p-Vehicle vs BTRA	<0.01
p-Vehicle vs CDS-P	<0.01
p-Vehicle vs CDS-PP	<0.01
p-KTP vs CDS-PA	<0.01
p-KTP vs BTRA	<0.01
p-KTP vs CDS-P	<0.01
p-KTP vs CDS-PP	<0.01
p-CDS-PA vs BTRA	0.35607
p-CDS-PA vs CDS-P	<0.01
p-CDS-PA vs CDS-PP	<0.01
p-BTRA vs CDS-P < 1 hr	0.075893
p-BTRA vs CDS-P > = 1 hr	<0.01
p-BTRA vs CDS-PP	<0.01
p-CDS-P vs CDS-PP	<0.01

the maximum responses, but there was no significant difference between the two across the time course tested (two-factor anova with replication, p=0.3561).

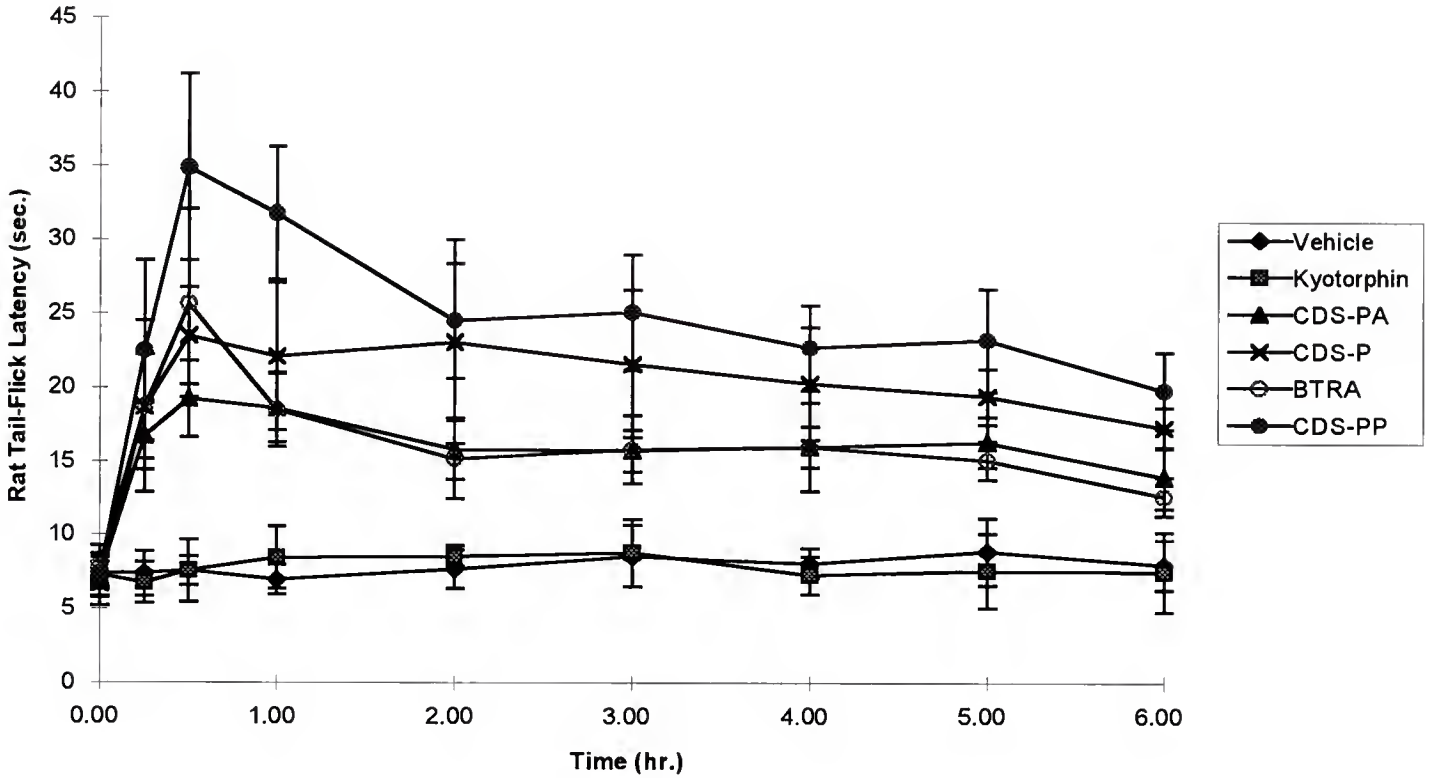
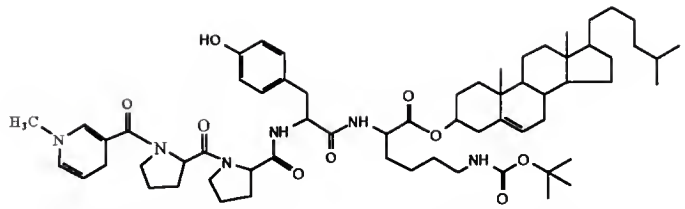


Figure 25. The Time Response of Rat Tail-Flick Latency of KAYK-CDS's and BTRA after i.v. Administration

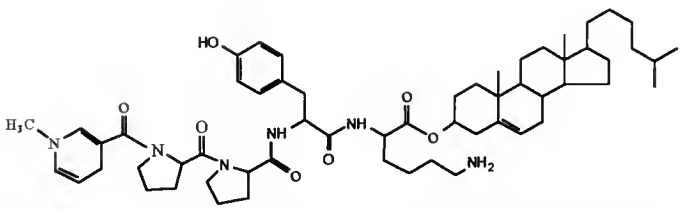
* Data represents mean ± SD of 6 rats.

The effects of CDS-PP and its four major intermediates (Figure 26) were investigated (Table 11 and Figure 27). Of the four intermediates tested, only DPPYKC, which is similar to CDS-PP except lacking the ε-Boc protection group at lysine, showed slight but significant analgesic effect over the vehicle and kyotorphin, but the potency was still much less than that of CDS-PP (single-factor anova for the maximum responses, $p<0.01$). Therefore, it is proved that both the dihydro and the cholesterol moieties are essential for the proper function of the CDS, and the ε-protection group on lysine is also a very important part.

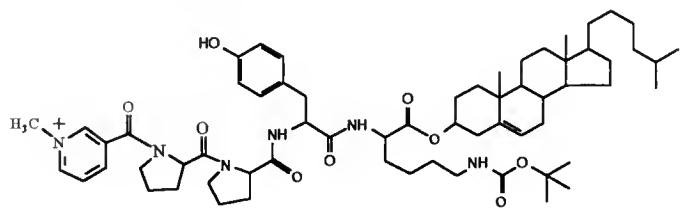
The effects of BTRA and its four major intermediates (Figure 28) are shown in Table 12 and Figure 29. Similarly, of the four intermediates tested, only YNC, which is similar to BTRA except lacking the α-Boc protection group at tyrosine, showed significant analgesic effect over the vehicle and kyotorphin, but the potency was still significant less than that of BTRA (single-factor anova for the maximum response, $p<0.01$). Moreover, the duration of its effect was also much less than that of BTRA. In other words, it did not display the sustained release desired (Table 12, Figure 30; two-factor anova with replication, $p<0.05$ vs BTRA, $p=0.60$ vs vehicle). The reason is probably due to the fact that the exposed α-amine terminal of the tyrosine of YNC makes the molecule vulnerable to various peptidases, especially aminopeptidases, present inside the brain.



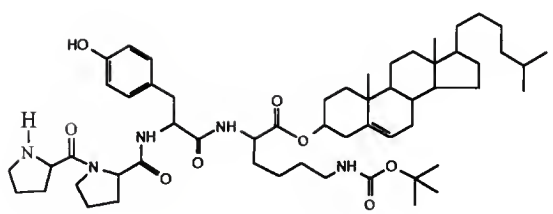
1,4-Dihydrotrigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester -- CDS-PP



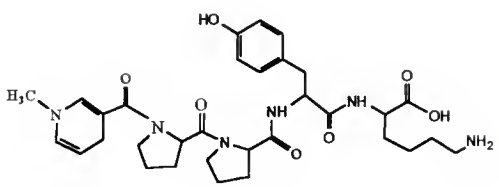
1,4-Dihydrotrigonellyl-Pro-Pro-Tyr-Lys-Cholesteryl Ester -- DPPYKC



Trigonellyl-Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester -- TPPYK(B)C



Pro-Pro-Tyr-Lys(Boc)-Cholesteryl Ester -- PPYK(B)C



1,4-Dihydrotrigonellyl-Pro-Pro-Tyr-Lys -- DPPYK

Figure 26. The Structures of CDS-PP and Its Major Intermediates

Table 11. The Rat Tail-Flick Latency Periods Prior To and After i.v. Administration of CDS-PP and Its Major Intermediates

	The Rat Tail-Flick Latency Periods (sec.)							
Time	Vehicle	KTP	KAYK	CDS-PP	DPPYKC	DPPYK	TPPYK(B)C	PPYK(B)C
0 min	7.4±1.1	7.3±1.4	8.4±1.3	7.4±1.1	7.6±1.6	8.1±1.6	8.3±1.9	7.8±1.4
15 min	7.4±1.5	6.8±1.4	8.8±1.0	22.5±6.1	10.3±1.3	10.1±2.0	7.9±1.3	8.7±1.2
30 min	7.6±1.0	7.6±2.1	8.8±1.2	34.9±6.3	11.9±1.8	10.2±1.2	8.0±1.0	8.8±1.2
1 hr	7.0±1.0	8.5±2.1	9.8±1.6	31.8±4.5	12.4±2.3	10.2±2.3	8.2±1.0	9.4±1.2
2 hr	7.7±1.3	8.6±0.7	9.4±1.4	24.5±3.9	11.3±1.7	8.7±1.4	9.9±1.4	8.5±1.2
3 hr	8.6±2.1	8.8±2.3	9.6±1.1	25.1±3.9	13.5±2.9	9.2±1.1	9.8±1.9	8.8±1.2
4 hr	8.1±1.0	7.3±1.3	8.6±1.5	22.7±2.9	13.9±2.3	8.5±1.1	8.9±1.6	8.5±0.8
5 hr	8.9±2.3	7.6±2.5	8.3±1.4	23.2±3.5	11.7±2.1	8.9±1.4	8.7±1.6	8.4±1.6
6 hr	8.0±1.7	7.5±2.7	7.9±1.2	19.8±2.6	10.0±2.1	8.1±1.2	7.7±1.2	7.4±0.9
Maximum responses	10.1±1.4	10.4±1.5	10.6±1.3	36.8±5.0	14.8±2.2	11.5±1.5	10.9±1.4	10.1±0.9
p-vehicle		0.4821	0.5319	<0.01	<0.01	0.2962	0.2962	0.9237
p-DPPYKC	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01

[a] Data represents mean ± SD of 6 rats. [b] p: single-factor anova

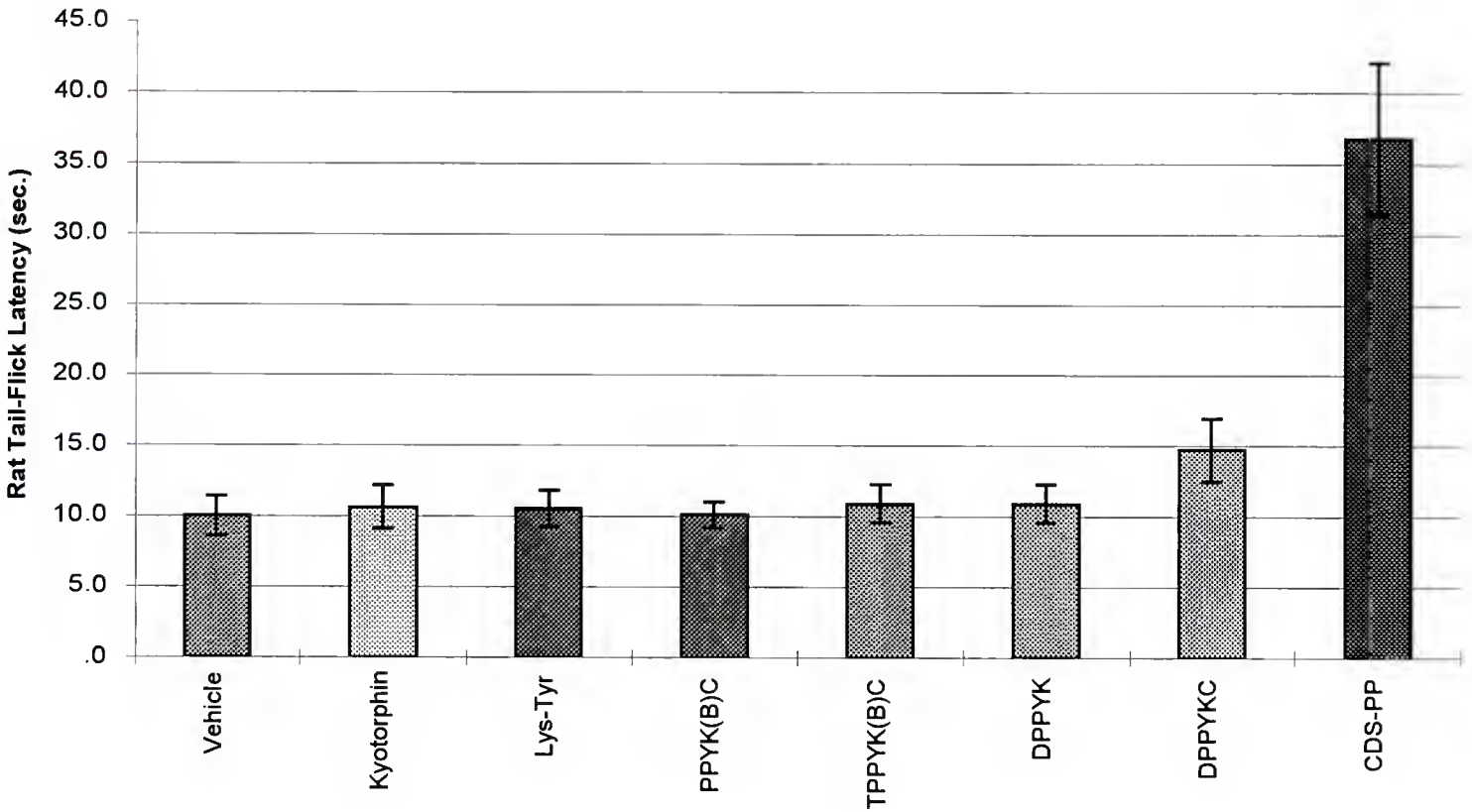
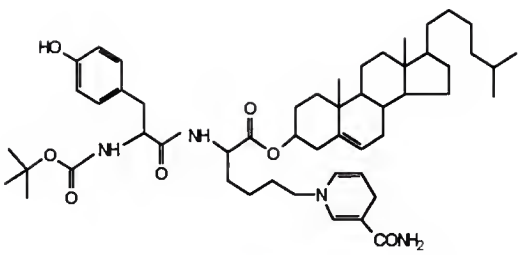
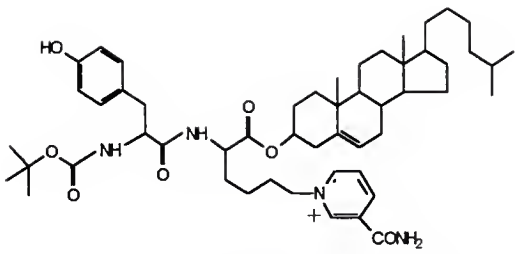


Figure 27. The Rat Tail-Flick Latency Periods After i.v. Administration of CDS-PP and Its Major Intermediates

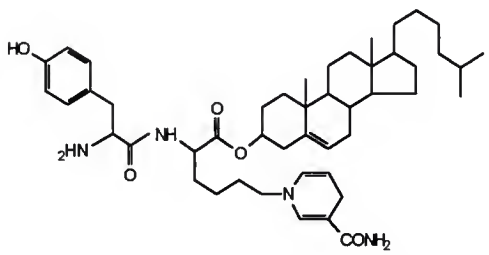
* Data represents mean ± SD of 6 rats.



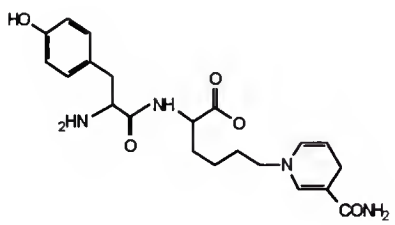
Boc-Tyr-Nys-Cholesteryl Ester -- BTRA



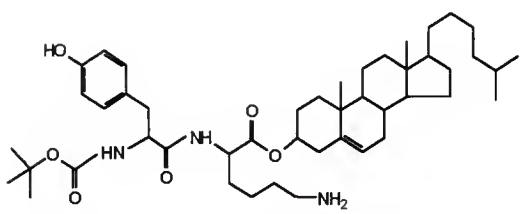
Boc-Tyr-Nys-Cholesteryl Ester -- BYN



Tyr-Nys-Cholesteryl Ester -- YNC



Tyr-Nys -- YN



Boc-Tyr-Lys-Cholesteryl Ester -- BYKC

Figure 28. The Structures of BTRA and Its Major Intermediates

Table 12. The Rat Tail-Flick Latency Period Prior to and After i.v. Administration of BTRA and Its Major Intermediates [a, b, c]

	The Rat Tail-Flick Latency Period (sec.)							
Time	Vehicle	KTP	KAYK	BYKC	$BYN^{+}C$	YN	YNC	BTRA
0 min	7.4±1.1	7.3±1.4	8.4±1.3	7.7±1.1	8.0±1.2	8.3±1.3	6.7±1.0	8.3±1.6
15 min	7.4±1.5	6.8±1.4	8.8±1.0	8.2±0.8	8.3±1.1	8.0±1.7	16.0±1.0	18.7±5.8
30 min	7.6±1.0	7.6±2.1	8.8±1.2	8.2±1.0	7.9±1.1	8.9±1.7	16.6±1.6	25.7±6.4
1 hr	7.0±1.0	8.5±2.1	9.8±1.6	8.9±1.3	8.9±0.9	8.9±1.5	9.6±1.4	18.5±2.5
2 hr	7.7±1.3	8.6±0.7	9.4±1.4	7.7±0.9	9.0±1.3	8.7±1.4	7.9±1.6	15.2±2.7
3 hr	8.6±2.1	8.8±2.3	9.6±1.1	8.5±1.2	8.9±1.3	9.1±1.2	7.5±1.3	15.8±2.3
4 hr	8.1±1.0	7.3±1.3	8.6±1.5	8.4±0.9	8.4±1.7	7.9±1.0	8.0±1.5	16±3.0
5 hr	8.9±2.3	7.6±2.5	8.3±1.4	8.2±1.5	8.9±1.0	8.4±1.3	8.3±1.3	15.1±1.3
6 hr	8.0±1.7	7.5±2.7	7.9±1.2	7.9±1.0	7.5±0.7	7.6±0.9	7.8±0.9	12.6±1.3
Maximum Responses	10.1±1.4	10.4±1.5	10.6±1.3	9.9±1.0	10.2±0.8	9.9±1.1	16.4±1.3	26.1±6.0
p-vehicle		0.4827	0.5319	0.7990	0.8817	0.8773	<0.01	<0.01
p-YNC	<0.01							<0.01
p_2-YNC < 1 hr	<0.01							<0.01
p_2-YNC > = 1 hr	0.6000							<0.01

[a] Data represents mean ± SD of 6 rats.
[b] p: single-factor anova.
[c] p_2: two-factor anova with replication.

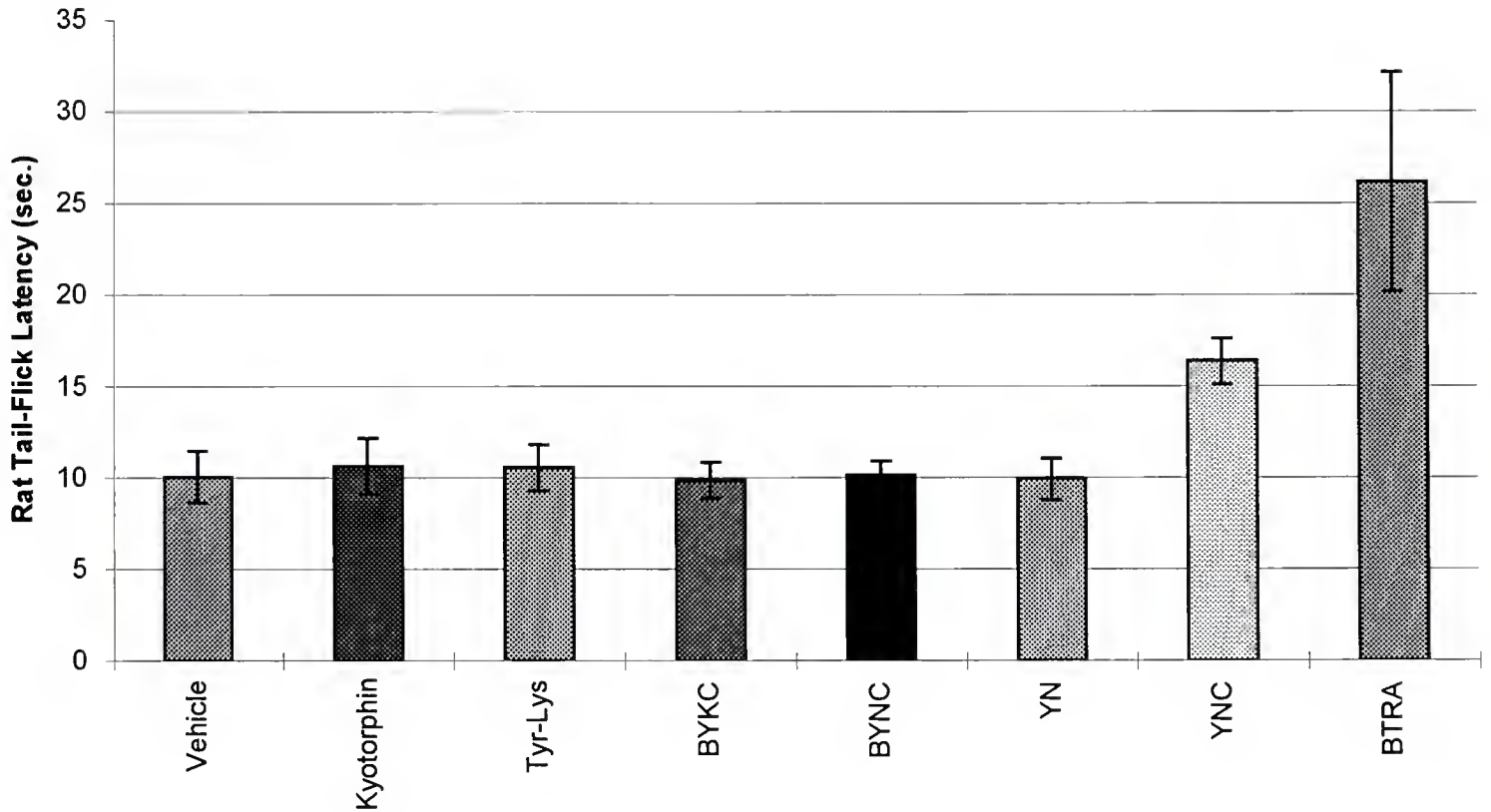


Figure 29. The Rat Tail-Flick Latency Periods After i.v. Administration of BTRA and Its Major Intermediates

* Data represents mean ± SD of 6 rats.

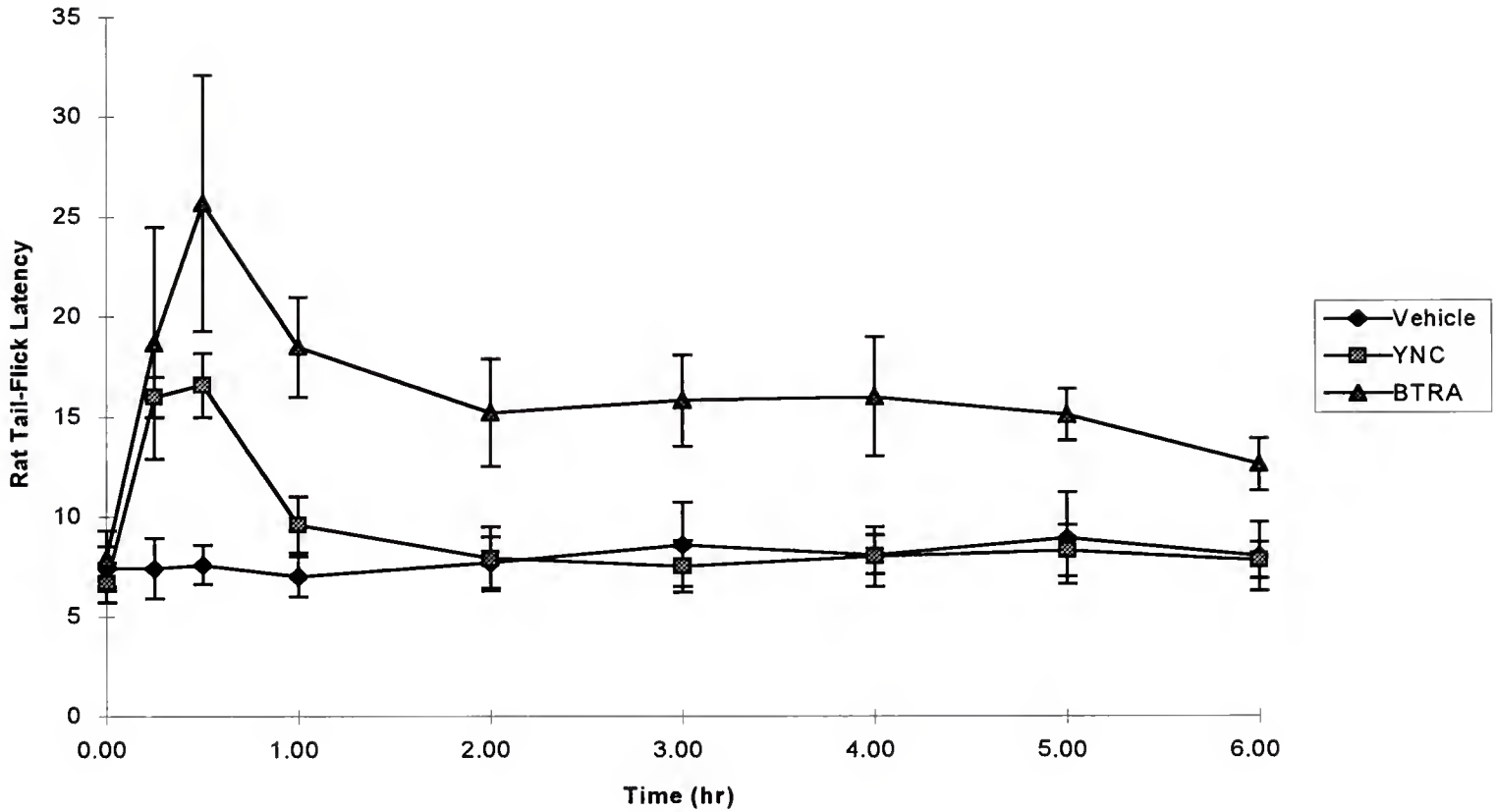


Figure 30. The Time-Response Relationships of BTRA and YNC after i.v. Administration

* Data represents mean ± SD of 6 rats.

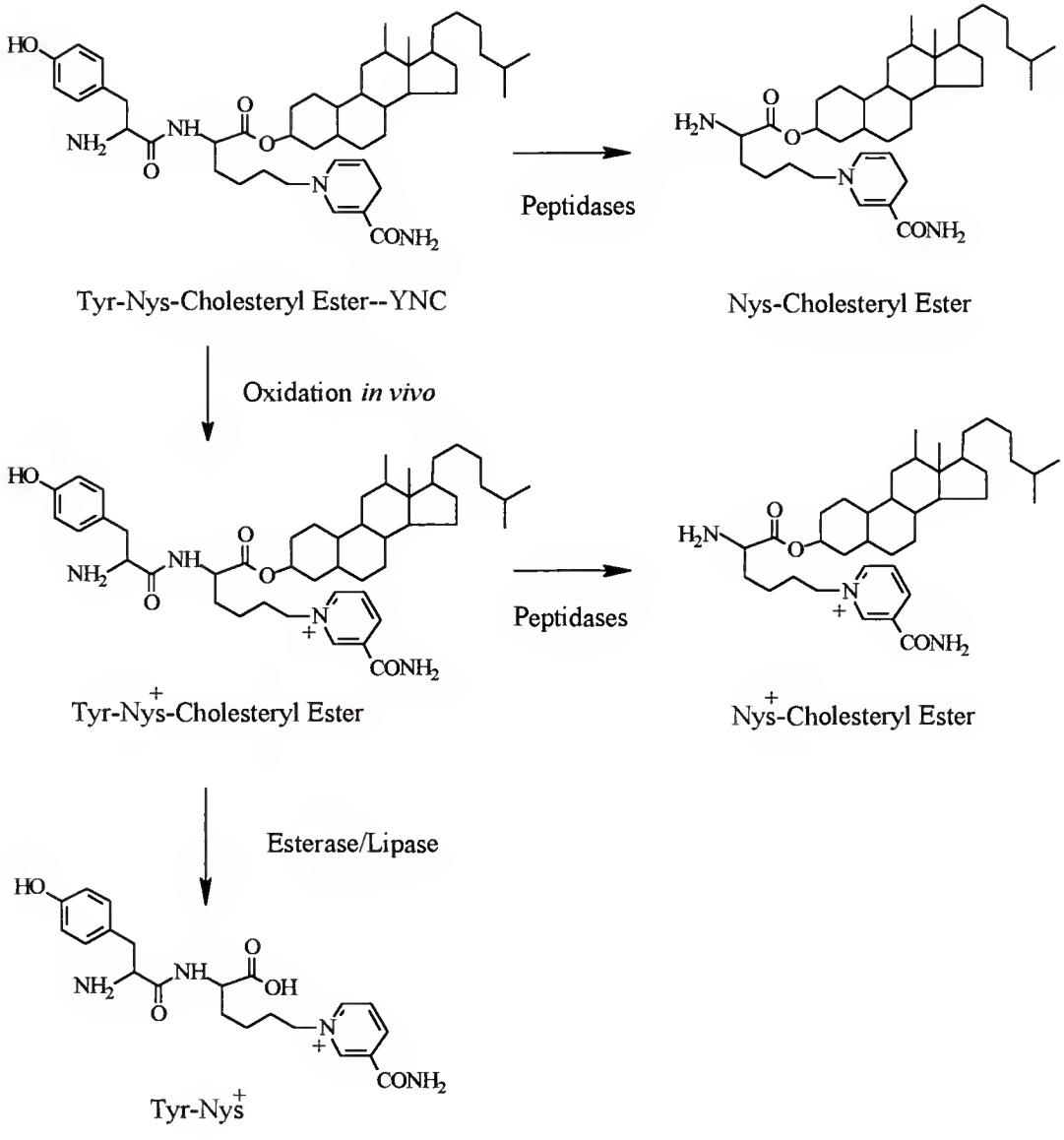


Figure 31. The Bioconversion of YNC *in vivo*

Therefore, both the dihydro and the cholesterol moieties are proved to be essential for the proper function of BTRA, and the importance of the α-protection group on tyrosine is also demonstrated.

Naloxone is a competitive antagonist at μ, δ, κ, and σ-opioid receptors. Small doses (0.4 to 0.8 mg) of naloxone given intramuscularly or intravenously in man prevent or promptly reverse the effects of μ-opioid agonist. In patients with respiratory depression, there is an increase in respiratory rate within one or two minutes. Sedative effects are reversed, and blood pressure, if depressed, returns to normal. One milligram of naloxone given intravenously completely blocks the effects of 25 mg of heroin. The duration of the antagonistic effects depends on the dose but is usually 1 to 4 hours.

Testing the effect of naloxone in rats treated with the KAYK brain-targeted delivery systems is important, since mechanism of the analgesic effect induced by the brain-targeted delivery systems of KAYK can be explained based on the fact that the CNS mediated analgesia is naloxone reversible and a peripheral mediated analgesia may not be reversed by naloxone.

Our studies indicated that the analgesic effects induced by CDS-PP and BTRA can be reversed by naloxone (Table 13, Figure 32; two-factor anova with replication, $p < 0.01$). The results suggested that the brain-targeted delivery systems

Table 12. The Effects of Naloxone on Rat Tail Flick Latency Periods After i.v. Administration of CDS-PP and BTRA [a, b, c]

	The Rat Tail Flick Latency Period (sec.)			
Time (hr.)	BTRA/N	CDS-PP/N	BTRA	CDS-PP
0.00	7.3±1.5	7.4±1.0	8.3±1.6	7.4±1.1
0.25	18.7±1.7	25.0±4.7	18.7±5.8	22.5±6.1
0.50	25.3±3.1	32.6±3.7	25.7±6.4	34.9±6.3
0.75	9.0±1.1	10.2±1.6		
1.00	7.5±0.8	8.9±1.2	18.5±2.5	31.8±4.5
1.50	7.6±0.9	8.0±0.7		
2.00	7.4±0.7	7.7±1.3	15.2±2.7	24.5±3.9
p-BTRA vs BTRA/N	<0.01			
p-CDS-PP vs CDS-PP/N				<0.01

[a] Data represents mean ± SD of 6 rats.

[b] Naloxone was administered s.c. at the dose of 2 mg/kg 30 minutes after the administration of CDS-PP and BTRA in the case of BTRA/N and CDS-PP/N.

[c] The rat tail-flick latency periods for CDS-PP and BTRA were not recorded at 0.75 hr and 1.5 hr.

[d] p: two-factor anova with replication.

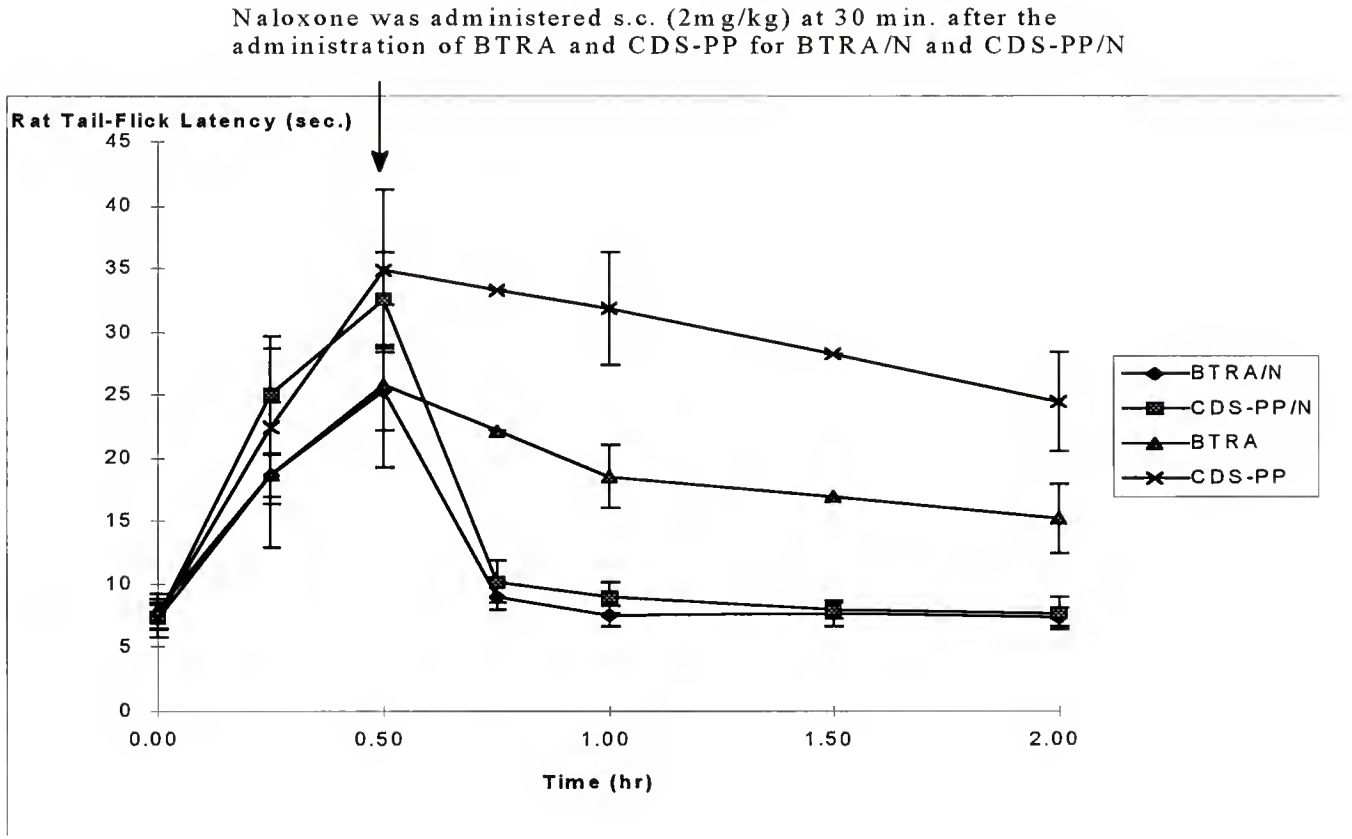


Figure 32. The Effects of Naloxone on CDS-PP and BTRA Induced Rat Tail Flick Latency Periods Increases

*: Data represents mean ± SD of 6 rats.

**: The tail-flick latency periods for CDS-PP and BTRA were not recorded at 0.75 hr and 1.5 hr.

of the KAYK could effectively deliver the Tyr-Lys and Tyr-Nys into the CNS and induce the CNS mediated analgesia.

The signal feature of opioid drugs is their ability to induce tolerance and dependence when given chronically to humans or experimental animals. Tolerance may be defined as a state in which the dose of drug required to achieve a given effect is larger than normal. Dependence is a state in which regular doses of the drug are required to prevent withdrawal symptoms.

Opioid dependence or addiction is, of course, one of the major health problems of our time, and a hindrance to the effective use of opioids as clinical analgesics. Increasing evidence suggests that changes in opioid receptors, as well as in certain other functional macro molecules with which they are closely associated, are involved in opioid tolerance and dependence. Although it is unlikely that any simple models can account entirely for such a complex phenomenon like opioid tolerance and dependence, it appears that the changes in receptors may play a key role in the process.

As with stimulation-produced analgesia, several studies suggest that Chinese traditional acupuncture analgesia, which does not induce any tolerance and dependence effects, may be mediated, at least in part, by endogenous opioid peptides (Mayer *et al.*, 1977). Kyotorphin, unlike other opioid peptides, does not bind to any opioid receptors and exhibits analgesic action by mediation of the release

of endogenous enkephalins from nerve terminals inside the brain (Takagi *et al.*, 1979). It was also reported that kyotorphin is lower in concentration in patients with persistent pain, which suggests that kyotorphin acts as a putative neuromediator and/or an endogenous pain modulator in the human brain (Nishimura, 1991).

The brain-targeted delivery system for KAYK offers us a whole new way to deliver the KAYK into the brain. More importantly, it has the sustained analgesic effect which is quite different from that of morphinoids. This controlled, sustained release of KAYK inside the CNS modulates other endogenous opioid peptides in a way which is different from any other known opioid-like drugs do, which may lead to a new generation of clinical analgesics that have opioid-like analgesia but do not have or have less opioid tolerance and dependence.

CHAPTER 5
CONCLUSION

One obstacle in the studies of the opioid peptides and their analogs is that they are not effective when administered systemically. This is due to the fact that they cannot cross the BBB to enter the CNS -- their site of action. The brain-targeted delivery systems, such as CDS and BTRA, designed by Dr. Bodor by using the molecular packaging is the first rational drug design approach for peptide brain delivery.

The fundamental features of the brain-targeted delivery systems designed by Dr. Bodor is as follows: 1) disguising the peptide nature of the parent peptide to confuse the peptide-degrading enzymes; 2) lipophilic enough to cross the BBB; 3) retention in the brain or "lock in" once the molecule crossed the BBB; 4) release of the biological active peptide according to a prescribed route *in situ*. The overall strategy is based on structural, physico-chemical and enzymatic aspects of the BBB.

Based on the "Molecular Packaging" concept, a series of chemical delivery systems and a brain-targeted redox analog for Tyr-Lys were synthesized. This was performed by a stepwise procedure in solution started with esterification of lysine derivatives with cholesterol and followed by coupling of the respective peptide cholesteryl ester with Fmoc-amino acids using DCC/HOBt method.

Analgesic function of the CDS's and BTRA was observed by the increased tail-flick latencies of rats, which indicated the successful delivery of Tyr-Lys into the brain. All CDS's showed analgesic activity upon systemic administration. CDS-PA, a CDS for Tyr-Lys with Pro-Ala as the spacer, increased the rat tail-flick latency more than 200%; CDS-P, a CDS for Tyr-Lys with proline as the spacer, increased the rat tail-flick latency more than 250%; CDS-PP, a CDS for Tyr-Lys with Pro-Pro as the spacer, increased the rat tail-flick latency more than 350%; and BTRA increased the rat tail-flick latency more than 250%. The induced analgesia can be reversed by naloxone.

The pharmacological study also suggested the following findings:

1) The Boc protection of amine groups in the brain-targeted brain delivery systems is bio-reversible when the desired compounds are inside the CNS.

2) Lysine redox analog can be a proper replacement for Arg and Lys for brain delivery. Lysine redox analog designed by Dr. Bodor is a completely new amino acid analog. Hence Tyr-Nys is a novel kyotorphin analog which exhibits analgesic effect. The fact that Nys can be used as a replacement for Arg and Lys will greatly facilitate the design of delivery systems for peptide containing basic amino acids and simplify their synthesis.

Morphinoids are still the primary clinical choice in the treatment of certain moderate to severe pain. The development of tolerance and physical dependence with repeated use is a characteristic feature of all the opioid drugs used today, and the possibility of developing drug dependence is one of the major limitations of their clinical

use. It is highly desired to develop an effective pain-reliever with morphine-like analgesia, but without the side effects. The brain-targeted delivery system for KAYK offers controlled, sustained release of KAYK inside the CNS that modulates other endogenous opioid peptides in a way which is different from any other known opioid-like drugs do. The brain-targeted delivery system for KAYK showed expected analgesic effects which may be used as a step stone for us to develop a new generation of clinical analgesics that induce opioid-like analgesia but not tolerance and dependence.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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